Rita Severino dos Loios

Bachelor Degree in Cellular and Molecular Biology

Optimization of strategies for anti-cancer vaccines

Dissertation to obtain the Master Degree in
Molecular Genetics and Biomedicine

Supervisor: Professor Doctor Paula Videira, FCT/UNL
Co-supervisor: Doctor Tiago João Ferro, FCT/UNL

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President: Prof. Doctor José Paulo Sampaio
Argue: Prof. Doctor Maria de Guadalupe Cabral
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FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Optimization of strategies for anti-cancer vaccines

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FCT-UNL, UNL

“If I cannot do great things, I can do small things in a great way”

M. Luther King

Acknowledgments

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Abstract

Nowadays, cancer is one of the leading causes of death worldwide and treatments currently in use are often aggressive, invasive and not very effective. Promising therapeutic approaches include immunotherapies that has the potential to boost the immune system to fight cancer.

One type of immunotherapy is provided by vaccines of dendritic cells (DCs) or containing artificial antigen presenting cells (aAPCs). These vaccines are used to expand and activate T cells against specific tumors antigens, eliminating tumor cells and providing immunological protection. Activation of T cells *in vivo* is lead mainly by DCs. aAPCs are artificial systems that have as main goal to mimic the function that DCs have *in vivo*, by presenting antigens, the first signal required for T cell activation. Vaccines of aAPCs are easier to produce and maintain and overcome the issues of patient-derived autologous DCs.

The main aim of this thesis was to implement an *in vitro* assay to test the potential of a new generation of aAPCs, named PEMs, to activate antigen specific human T cells. PEMs consist of carbonated cores assembled with HLA-A02 molecules loaded with peptides.

To verify PEMs ability to activate T cells against a specific antigen (cytomegalovirus (CMV) peptide), PEMs were co-cultured with human $CD8^+$ T cells, isolated from healthy donors. The activation of the cells was verified by the measure of the expression of IFN-γ by qPCR and ELISA.

Results showed that, in some donors, there is an increase of IFN-γ when T cells are stimulated with PEMs complexed with HLA-A02 loaded with CMV peptide, compared with negative controls. PEMs show significant binding to $CD8^+$ T cells.

With this study it was possible to conclude that PEMs are able to bind and activate T cells. However, there are still issues related to their stability and specificity. Future studies testing other aAPCs that present distinct cores and assemblies should be performed.

Keywords: Cancer; Immunotherapies; T cells; APCs; aAPCs

Resumo

Atualmente, o cancro é uma das principais causas de morte no mundo e os principais tratamentos que são utilizados são frequentemente agressivos, invasivos e pouco eficazes. Abordagens terapêuticas promissoras incluem imunoterapias que têm o potencial de impulsionar o sistema imunológico para combater o cancro.

Um tipo de imunoterapia é fornecido por vacinas de células dendríticas (DCs) ou que contém células artificiais apresentadoras de antigénios (aAPCs). Estas vacinas são usadas para expandir e ativar as células T contra antigénios tumorais específicos, eliminando células tumorais e fornecendo proteção imunológica. A ativação de células T *in vivo* é conduzida principalmente por DCs. aAPCs são sistemas que têm como principal objetivo imitar a função que as DCs têm *in vivo*, apresentando antigénios, o primeiro sinal necessário para a ativação das células T. Vacinas de aAPCs são mais fáceis de produzir e manter e permitem ultrapassar os problemas de DCs autólogas derivadas de pacientes.

O principal objetivo desta tese foi implementar um ensaio *in vitro* para testar o potencial de uma nova geração de aAPCs, denominadas PEMs, para ativar células T humanas específicas para determinados antigénios. PEMs consistem em núcleos de carbono ligados a moléculas de HLA-A02 carregadas com péptidos.

Para verificar a capacidade das PEMs para ativar células T contra um antigénio específico (péptido do citomegalovirus (CMV)) as PEMs foram co-cultivadas com células T $CD8^+$ humanas, isoladas de dadores saudáveis. A ativação das células foi verificada pela medição da expressão de IFN-γ por qPCR e ELISA.

Os resultados mostraram que, em alguns dadores, houve um aumento de IFN- quando as células T foram estimuladas com PEMs complexadas com moléculas de HLA carregadas com o péptido CMV, em comparação com os controlos negativos. PEMs mostram ligação significativa às células T $CD8^+$.

Com este estudo foi possível concluir que as PEMs podem ligar-se e ativar células T. Contudo ainda existem questões relacionadas com a sua estabilidade e especificidade. Futuros estudos, que testem outras aAPCs que apresentem outros núcleos e formas devem ser realizados.

Palavras-chave: Cancro; Imunoterapias; Células T; APCs; aAPCs

Table of Contents

Index of Figures

Index of Tables

List of Abbreviations

aAPC's	Artificial antigen presenting cells
ALP	Alkaline phosphatase
APC	Allophycocyanin
APCs	Antigen presenting cells
CAR	Chimeric antigen receptor
cDNA	Complementary deoxyribonucleic acid
CLIP	Class II-associated invariant chain peptide
CMV	Cytomegalovirus
Ct	Threshold cycle
CTLA 4	Cytotoxic T lymphocyte associated antigen 4
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HTLV	Human T-lymphotropic virus
IFN	Interferon
IL	Interleukin
Ion	Ionomycin
IPST	Instituto Português do Sangue e da Transplantação
LPS	Lipopolysaccharide

MHC	Major histocompatibility complex
MHC I	Major histocompatibility complex, class I
MHC II	Major histocompatibility complex, class II
mRNA	Messenger ribonucleic acid
NK	Natural killer
PAA	Poly(acrylic acid) sodium salt
PAH	Poly(allylamine hydrochloride)
PAMPs	Pathogen-Associated Molecular Patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEMs	Polyelectrolyte microcapsules
PerCp	Peridinin chlorophyll protein complex
PLGA	Poly(lactic-co-glycolic acid)
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern-Recognition Receptors
qPCR	quantitative PCR
RNA	Ribonucleic acid
RNase	Ribonuclease
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcriptase polymerase chain reaction
Sulfo-NHS	Sulfo N-Hydroxysuccinimide
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TLRs	Toll-like receptors
TMB	3,3',5,5'-tetramethylbenzidine
TNF	Tumor necrosis factor

1 Introduction

1.1 Immunity, immune system and immunology

Immunity is defined as resistance to diseases, more specifically to infectious diseases [1].

The immune system involves many organs, molecules, cells and pathways in interconnected processes and has evolved as the host's defense, to protect multicellular organisms from pathogens and cancer [2], [3].

Immunology is the study of the immune system and the responses to the invading pathogens microorganisms. The reaction of the components of the immune system against infectious microorganisms is known as the immunologic response [1].

In vertebrates the immunologic defenses are divided into two immunologic subsystems – the innate and adaptative immune responses [4].

1.1.1 Innate immunity

Innate immunity is the first line of defense and it is found in all multicellular plants and animals [5]. It works rapidly (in some hours) against the infectious insult and gives rise to the acute inflammatory response. When a host is attacked by a pathogen it is the innate immune system that responds with immediate action. In the innate immune system are included the epithelial barriers, specialized cells and natural antibiotics that block the entry of the microorganisms in the epithelia [2]. In the tissues or in the circulation, the microorganisms are destroyed by the phagocytes (macrophages and neutrophils), the natural killer (NK) cells and also plasmatic proteins, where the complement system is included. This type of immunity has some specificity for microbes but no memory [5], [6].

The innate immune system recognizes the pathogens through pattern-recognition receptors (PRR). These receptors identify particular types of molecules that are common to many pathogens, known as pathogen-associated molecular patterns (PAMPs), that do not exist in the host [7], [8]. The lipopolysaccharide (LPS), a component of the Gram-negative bacteria cell wall (recognized by the toll-like receptor 4 (TLR-4)) and the teichoic acids on Gram-positive bacteria are examples of PAMPs [8], [9]. Different PRR recognize specific PAMPs which will activate specific signaling pathways leading to different responses against pathogens [7], [8].

1.1.2 Adaptive immunity

In addition to the innate immune responses, vertebrates also have an adaptive immunity. The adaptive immune responses are the second line of defense and are highly specific to a particular antigen [10]. This type of immunity takes more time to be effective, usually days, since the organism is first exposed to the antigen, but provides long-lasting protection mediated by cells and molecules forming the immunologic memory. Innate immune responses are responsible for

activating adaptive immunity and together they work in order to eliminate the foreign molecules. B and T lymphocytes are white blood cells that are responsible for the adaptive immune responses. There are two types of adaptive immunity, the humoral responses and the cell-mediated immune responses [10]–[12].

B cells are involved in the humoral responses. In this type of response, B cells are activated and differentiate into plasma cells, producing antibodies, which are proteins that will destruct microorganisms in extracellular spaces. Activation of B cells is stimulated by specific soluble antigens, and the antibodies produced specific for that antigens. The antibodies binds to the pathogens blocking them or mark them for destruction that is carried out mostly by phagocytic cells [10][13].

T cells are responsible for cell-mediated immune responses. T cells are activated when antigen presenting cells (APCs) present the antigen to the T cell in the context of major histocompatibility complex (MHC). Dendritic cells are professional APCs which ingest the antigens and then present them. Activated T cells can destroy directly the infected cells (effector T cells) or they can produce molecules that will activate macrophages and promote the phagocytosis (helper T cells) [10][14].

1.1.3 T cells

Lymphocytes are developed in lymphoid organs. T lymphocytes are derived from stem cells in the bone marrow but their development occurs mainly in the thymus. The immature T cells derived from bone marrow migrate towards the thymus where maturation takes place. Interactions between immature T cells and thymic stromal cells lead to the differentiation and posterior proliferation of T cells. The development of T cells occurs in different phases and in each phase occurs changes at T cell receptor (TCR) genes and expression levels. [15][16]

TCR is a disulfide-linked heterodimeric protein that is attached to the membrane. These protein is typically constitute by a complex with two different parts. One part consisting of the highly variable α and β chains and the other part is constitute by the invariant CD3 chain molecules.[16]

Majority of T cells express the α and β chains (α:β T cells) but there is a minority that express an alternate receptor that is formed by variable chains γ and δ (γ:δ T cells). During TCR development occurs a specific process of gene recombination in somatic T cells. TCRα gene locus contains variable (V) and joining (J) gene segments and TCRβ contains, beside V and J segments, a D (diversity) gene segment. Therefore, α chain is generated from VJ recombination and β chain from VDJ recombination. In γ:δ TCRs the process is similar since VJ recombination is involved in γ and δ chain is generated from VDJ recombination. [17], [18]

During maturation, cell-surface proteins expression is altered, mainly involving the TCR (CD3) complex expression and CD4 and CD8 proteins. Mature T cells express a diverse of antigen specific receptors - T cell receptors, due to the rearrangement of the genes that encode

to the antigen receptors and the changes of expression that happen in cell-surface and intracellular proteins. [15]

In the last phase of development, α:β T cells originate two types of cells - $CD4^+$ and $CD8^+$ T cells, which are functionally different. Cells where the rearrangements do not occur properly die by apoptosis.[16]

TCR recognize only antigens that are bound to MHC molecules in the surface of APCs. $CD4^+$ T cells are called helper cells because they provide signals and cues to B lymphocytes in antibody productions and to phagocytic cells in their function. $CD8^+$ T cells are named cytotoxic T cells since they kill infected or aberrant cells. [1]

1.1.4 T cell activation

To activate T cells, three different signals are required. Signal 1 occurs when an APC presents an antigen on the surface bound to MHC molecules, to be recognized by the TCR. Furthermore, CD4 molecules on helper T cells and CD8 molecules on cytotoxic T cells will bind to MHC II and MHC I on APC, respectively. This selective recognition is termed MHC restriction. These interactions between TCR and peptide-MHC complexes and between CD4 or CD8 and MHC II and MCH I will induce a cascade of signaling from the cytosol to the nucleus. This cascade of events involves many interactions and modifications on proteins, such as phosphorylation and ubiquitination, interacting with many second messengers, like calcium and diacylglycerol. It is a fact that TCR recognize the antigens that allow a specific response. [19]–[22]

Signal 2 is co-stimulation, where the costimulatory molecules from APC bind to co-receptors on the surface of the T cells. If co-stimulation does not occur, T cells can recognize the antigen but they cannot respond and die or enter in a state of anergy (unresponsive state) [20]. The best known T cell costimulatory pathway involves the costimulatory molecules B7-1 (CD80) and B7-2 (CD86) expressed on APCs and the CD28 receptor expressed on T cells. Pathogens and cytokines enhance the expression of costimulatory molecules in the surface of APCs. Signal 1 and 2 promote the expansion of T cells which were stimulated, and their subsequent differentiation in effector and memory cells. CD28 receptor induce the production of cytokines, especially of interleukin-2 (IL2). These cytokines promote metabolism and up-regulation of cell-survival genes [20], [21], [23].

However, there are also signaling molecules driving inhibitory responses. Cytotoxic T lymphocyte associated antigen 4 (CTLA-4) is one of those molecules and it can bind to B7-1 and B7-2, since it is a homologue of CD28. CTLA-4 is a negative regulator of T cell activation while it is expressed in activated T cells. When expression of CTLA-4 is upregulated, the expression of CD28 is downregulated by endocytosis and CTLA-4 can bind to the costimulatory molecules on APC and inhibit the T cell responses, by blocking the production of IL-2 and the progression of cell-cycle [20], [23].

Also, the expression of costimulatory molecules B7-1 and B7-2 is regulated on the APCs being dependent of the activation state of the cell. Transcription, translation and trafficking of B7-

1 and B7-2 to the surface of APCs are enhanced by the recognition of infection and stress and cellular levels by innate receptors. This regulation is essential to prevent aberrant and autoreactive T cell responses allowing the responses against non-self antigens [20].

Signal 3 is provided by cytokines produced by macrophages and dendritic cells, and is required for the effective production of effector and memory cells as well as for the survival of T cells. The knowledge about this third signal of stimulation is recent and the cells that do not receive this signal do not develop cytolytic function and stay unresponsive. Cytokines that provide signal 3 are essentially IL-12, interferon (IFN)-α, IFN-β and IL-21 [20], [24]. Figure 1.1 illustrates the three signals needed for T cell activation.

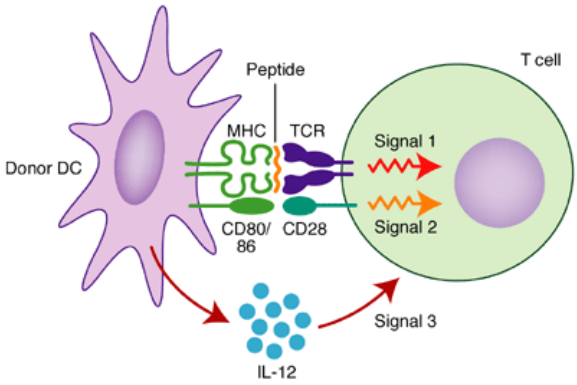


Figure 1.1 - Three signals for T cell activation. *Interaction between dendritic cells and T cells for T cell activation involves three signals. Signal 1 consists in the interaction between MHC containing peptide fragments on DC and the TCR on the T cell. Signal 2 is provided by the interaction between co-stimulatory molecules on DC surface and their receptors on T cell surface. Signal 3 occur when DC secrete IL-12. Adapted from* [25].

1.1.5 T cells differentiation after activation

After activation of T cells, they can differentiate essentially in two types: helper T cells (CD4) and cytotoxic T cells (CD8). Helper cells are responsible for production of cytokines, that activate other cells, and chemokines that recruit other cells to the place of infection. The main function of cytotoxic cells is the killing of pathogen infected cells by delivering cytotoxic granules in their cytosol. As there is a very diverse number of pathogens, the host produces different specialized T cells responses specific for the invading pathogen. Helper T cell responses are divided into different subsets depending on the pathogen, being the major ones Th1, Th2 and Th17 subsets. In the Th1 subset there is the production of large amounts of IFN-γ and tumor necrosis factor (TNF) α in response to intracellular pathogens such as virus. The Th2 subset is characterized by the production of large amounts of IL-4, IL-5 and IL-13 which will activate eosinophils, mast cells and basophils to eliminate parasites [26]. In order to eliminate extracellular bacteria and fungi, Th17 subset is activated and these cells produce IL-17. This cytokine is responsible for the activation of neutrophils which have the ability to kill bacteria and fungi [27]. Cytotoxic T cells are responsible for elimination of intracellular pathogens and cancer cells and they are activated after

they recognize their antigens in the presence of cytokines (IFN-α, IFN-β and IL-12). After activation cytotoxic T cells produce large amounts of IFN-γ, TNF-α and secretory vesicles that contain perforin and granzyme proteins that have the ability to kill the other cells and disseminate close to them [21].

1.1.6 Antigen presenting cells (APCs)

APCs are cells that are able to capture antigens, process them into peptides and present them to lymphocytes to activate them. DCs, macrophages and B cells present antigens mainly to T cells and are considered professional APCs since they express MHC II and co-stimulatory molecules [28]. However, there are other cells that can also present antigens such as thymic epithelial cells and vascular endothelial cells. In the lymph nodes, follicular dendritic cells act as APCs for B cells. APCs present antigens in form of peptide-MHC complexes. To present antigens to a $CD4^+$ T cell, the presentation is made in association with MHC II. If an antigen is to be presented to $CD8^+$ T cells, APCs do it via MHC I [28], [29].

DCs are the most potent because are the only APCs capable of activating naïve T cells, and also because they can present antigens either via class I or class II MHC molecules activating $CD4^+$ and $CD8^+$ T cells. [30] They also present a constitutive expression of B7 co-stimulatory molecules. Costimulatory molecules are essential to a properly activation of T cells as mentioned before. [28]

DCs capture antigens that invade the organism by phagocytosis, micropinocytosis or endocytosis[3]. DCs reside in certain areas of epithelial tissues and express receptors that bind directly to microorganisms and mannose residues on glycoproteins (bacterial characteristic). After recognition, the antigens are mainly captured by endocytosis. Besides endocytosis, antigens can bind also to TLRs on DCs and macrophages, enhancing the production of TNF and IL-1. Altogether, these factors contribute to activate DCs, inducing their migration towards lymph nodes in response to chemokines. In the lymph nodes, they can activate T cells. During migration DCs become mature and start to express costimulatory molecules and MHC molecules in a stable way [28], [31].

1.1.6.1 Antigen Processing and Presentation

To activate T cells, antigens need to be presented by APCs in form of peptides-MHC complexes or lipid-CD1 molecules complexes. In humans, MHC is designated by human leukocyte antigen (HLA). APCs express MHC II in their native state, while MHC I is expressed in all nucleated cells (including APCs). There are two major pathways responsible for the processing and presentation of antigens. One of the pathways is related to processing extracellularly-derived proteins (exogenous), presenting them via MHC II , while the other pathway presents cytosolic proteins (endogenous) via MHC I. [32]

However, DCs are also capable of process and present extracellular antigens via MHC I through cross-presentation. There are two main intracellular pathways for cross-presentation.[33] Cytosolic pathway involves the uptake of exogenous antigens and their transport from endosomal vesicles into the cytosol. In the cytosol, antigens are degraded by the proteasome and then transported by a transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER) where they are loaded on MHC I molecules.[34]–[36]

Vacuolar pathway is independent of proteasome since capture antigens are processed by endosomal proteases and load on MHC I in endosomal compartments. [34]–[36]

1.1.6.2 MHC II/Endocytic Pathway

Endocytic pathway (Figure 1.2) is responsible for processing the proteins that enter in the cell by endocytosis, phagocytosis or pinocytosis. These proteins are internalized in phagosomes, that fuse with lysosomes creating phagolysosomes where proteins are processed by proteolytic enzymes. This enzymatic cleavage generates a diverse array of peptides. [37]

MHC II molecules synthesized in the ER have the peptide-binding site blocked by the invariant chain (Ii). After synthesis, MHC II molecules are secreted in vesicles that posterior will fuse with the phagolysosomes where the peptides were processed. There the invariant chain is broken leaving only a small fragment called class II-associated invariant chain peptide (CLIP) that blocks the peptide-binding site. Then CLIP is removed by an MHC II-like structure and MHC II can bind to the peptides and migrate into exocytic vesicles to the cell surface in order to present the antigen to CD4 cells.[32], [38]

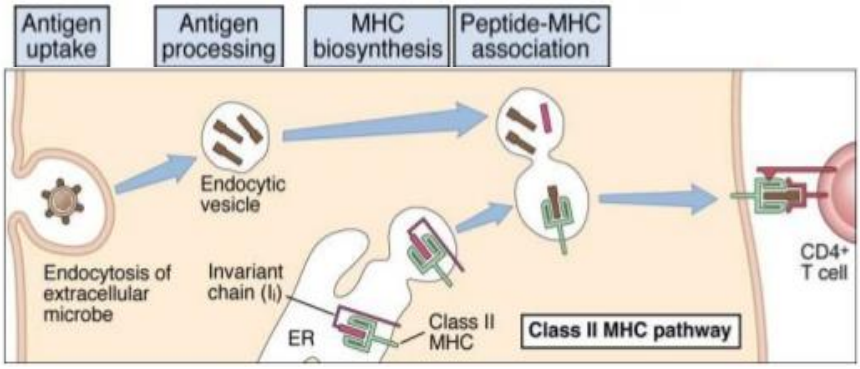


Figure 1.2 - MHC II/Endocytic pathway for intracellular processing of proteins antigens. *Protein antigens found in endocytic vesicles are converted into peptides that bind to MCH II molecules for recognition by CD4+ T cells. Adapted from* [5]

1.1.6.3 MHC I/Cytosolic Pathway

The cytosolic pathway (Figure 1.3) is responsible for processing proteins generated in the cytosol, such as viral proteins in infected cells or native proteins from the cell that are aberrantly synthesized or no longer needed. All these proteins are ubiquitinated and degraded in the

proteasome and then translocated by TAP from the cytosol to the ER, where they are combined with MHC I molecules.[39]

MHC I molecules, synthesized in the ER, are constitute by a polymorphic heavy chain and a chain called β2-microglobulin. In the ER, MHC I molecules without peptides are stabilized by chaperone proteins and the association of MHC I/β2-m heterodimers. When the right peptide finds the right MHC I, they form a non-covalent complex and MHC I becomes stable. The complex peptide-MHC I then migrates to the cell surface through the trans-Golgi network in order to present the antigen to $CD8^+$ cells [32], [38]

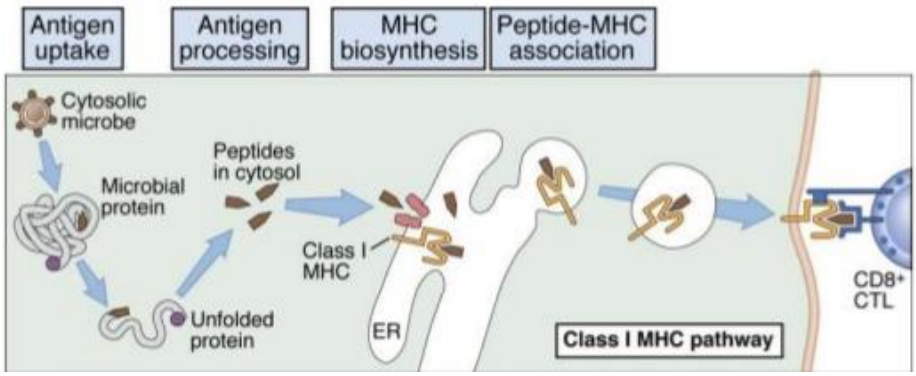


Figure 1.3 - MHC I/Cytosolic pathway for intracellular processing of proteins antigens. *Cytosolic proteins are converted into peptides that bind to MHC I molecules to be recognize by CD8+ T cells. Adapted from* [5]

1.2 Cancer

Cancer is one of the leading causes of death worldwide along with cardiovascular, chronic lower respiratory and neurodegenerative diseases and diabetes [40]. In general, 1 in 6 deaths are caused by cancer and it is expected that in the next two decades the number of new cases will increase by about 70%. Neoplasms of lung, liver, colorectal, stomach and breast are the most common [41]. Cancer results from genomic modifications in normal cells that turn them into tumor cells. These alterations consist on the accumulation of somatic mutations that lead to uncontrolled growth and proliferation of the mutated cells. Some of these alterations are related to the interaction between genetic factors and external factors, which include physical carcinogens (e.g. ultraviolet and ionizing radiation), chemical carcinogens (e.g. aflatoxin and arsenic) and biological carcinogens (e.g. infections by certain viruses, bacteria or parasites). More common mutations that lead to cancer are those that occur in genes responsible for deoxyribonucleic acid (DNA) repair, in proto-oncogenes and tumor suppressor genes. Mutations in proto-oncogenes lead to production of oncogenes with dominant gain of function and mutations in tumor suppressor genes lead to a recessive loss of function [40]–[42].

All these changes can occur in any cell of the body, leading to different types of cancer. Each neoplasm has a different behavior and that's why each type responds differently to treatments.

Tumor growth can be benign or malignant. In general, benign tumors remain confined to their original location; otherwise, in malignant tumors, abnormal cells can invade surrounding normal tissues and also spread to other organs via circulatory or lymphatic systems in a process called metastasization, being metastases the major cause of death from cancer [41].

Depending on the type of cell from which tumors arise they are classified into: carcinomas, originating from epithelial cells; sarcomas, that arise from connective tissues and are relatively rare in humans; leukemias that arise from blood forming cells and lymphomas that arise from immune system cells [42], [43].

Tumors consist of complex tissues formed by multiple distinct cell types that interact between themselves. Cancer cells and the tumor microenvironment contribute to many of the hallmark capabilities that tumors present. Cancer hallmarks (Figure 1.4) consist of acquired capabilities of cancer cells that allow them to survive and proliferate. Cancer cells can be self-sufficient in growth signals and insensitive to anti-growth signals because many cell survival and proliferation signaling pathways are deregulated, thus allowing cancer cells to maintain a chronic proliferation. These cells can avoid apoptosis may present a limitless replicative potential due to their ability to maintain telomeric DNA at lengths that avoid apoptosis. Angiogenesis, tissue invasion and metastasis are also typical abilities of tumor cells. Angiogenesis is triggered by hypoxia and allows cancer cells to obtain nutrients and oxygen. Other hallmarks may consist on evading the immune system through genetic and epigenetic modifications and reprograming energetic metabolism pathways, which is related to the fact that tumor cells can limit their energy metabolism to glycolysis even in the presence of oxygen leading to a state of aerobic glycolysis (Warburg effect) [44], [45].

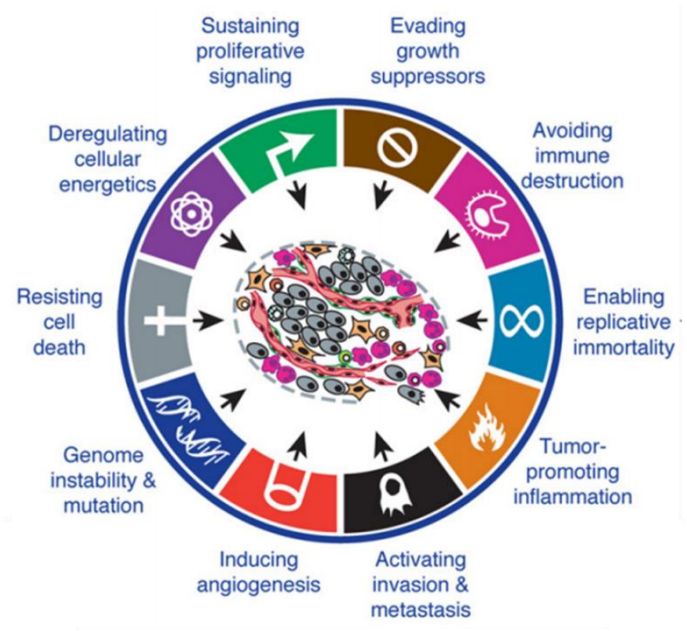


Figure 1.4 - Hallmarks of Cancer. *Functional capabilities acquired by cancer during their development. Adapted from* [44]

1.2.1 Cancer treatment

There are many types of treatment options for cancer patients and the treatment that each patient receives is related to the type of cancer and how advanced it is. Most of cancer patients receive a combination of treatments. Currently, combination of surgery with chemotherapy and/or radiotherapy is the most common treatment regime. Surgery is considered the best procedure for solid tumors that are confined to a specific area [46]. With surgery, it is possible to remove the entire tumor in some cases. In other cases, when the removal of the entire tumor can cause damage in an organ, the tumor should be debulked, which means that part of the tumor, but not all, is removed. Sometimes, surgery is only used to remove tumors that are causing pain to the patients [46]

Radiotherapy uses high doses of radiation to kill cancer cells and chemotherapy uses drugs to kill cancer cells. Radiotherapy and chemotherapy not only kill cancer cells, but these therapies also have the potential to kill healthy cells. This causes side effects like nausea, hair loss and mouth sores [47].

More recently, new and more specific therapies have been developed, such as immunotherapy, targeted therapy, hormone therapy and stem cell transplant. Immunotherapy is a biological therapy in which substances made from living organisms (or cells itself) are used to treat cancer. In immunotherapy, the host immune system is helped to fight cancer. Immunotherapies include the ones where cancer cells are marked to be easier for the immune system to find and kill them, usually by antibodies, and the ones where the immune system is boosted to be easier the fight against cancer. Targeted therapy consists of the design of essentially small molecule drugs or monoclonal antibodies which will target the changes in cells that lead to cancer and block their effects. Hormone therapy uses hormones in order to slow down or stop cancer from progressing. This type of therapy is essentialy used in prostate and breast cancers since they are hormone-sensitive to grow. In patients who receive systemic chemotherapy or radiotherapy to treat cancers, the blood cells are usually destroyed. In this case, a stem cell transplant can be performed in order to restore blood-forming stem cells, since all types of blood cells are important to the health of the organism. [47]

1.2.2 Cancer immunotherapy

Cancer immunotherapy involves the use of components of the immune system in order to treat cancer patients. Immunotherapy against cancer has the purpose to kill cancer cells by activating the immune system against these cells either passively or actively. Passive immunotherapy is related to, for example, monoclonal antibodies which are designed to recognize specific targets expressed by the tumor cells or existing in the tumor microenvironment. In this type of therapy, there is a higher specificity and off-target toxicities can be avoided. In contrast, active immunotherapy is related to the stimulation of immune cells to fight cancer. In this case, immune cells, especially T cells and B cells, are stimulated for a specific antigen. In fact, the knowledge that T cells are able to recognize specifically cancer cells and kill them is exploited in

this case. Accordingly, it is possible to produce a large number of activated T cells specifically against tumor antigens, eliciting the elimination of cancer cells. Adoptive T cell transfer is based on the same scheme: T cells isolated from cancer patients are stimulated with tumor antigens and expanded *ex vivo,* for later reinfusion into patients. After reinfusion, T cells will respond to cancer cells by killing them [48][49].

Chimeric antigen receptor (CAR) T-cell therapy is one specific type of adoptive T cell transfer therapy with leading results in the field and tested in small clinical trials. In this particular therapy, white blood cells are removed from patients and then T cells are separated and genetically modified. The modification consists of the addition of the specific CAR which will recognize a specific tumor antigen. After expansion of CAR T-cells, they are reintroduced into patients, specifically targeting cancer cells [49].

Cytokines, especially interferons and interleukins, are also used to treat cancer. These molecules are produced by the host own cells and have a key role in normal immune responses and in the response of the immune system against cancer [48].

Treatment vaccines against cancer are other type of immunotherapy. In this case there is a boost of the immune systems in order to respond to cancer cells [48].

One type of anti-tumor therapeutic vaccines is the one where patients are vaccinated with DCs, expanded and activated *ex vivo*, promoting and improving the activation of T cells. Since this process is expensive and time consuming, and the DCs generated can present a variable quality, other methods to activate T cells are starting to appear in the experimental setting. Artificial antigen presenting cells (aAPCs) are a new method that has been used to activate specifically T cells against tumor antigens either *ex vivo* or *in vivo* [50].

1.3 Artificial antigen presenting cells (aAPCs)

aAPCs are engineered systems for activation and expansion of T cells, mimicking the role of DCs. These systems include engineered cells or synthesized biomaterials and they have the advantage of being more controlled and reproducible [50], [51]. However it is very important to consider some variables when aAPCs are designed. These variables include the size, shape, mechanism for cytokine delivery and ligand distribution, mobility and positioning on aAPCs. All these factors will influence T cell activation. Most aAPCs are spherical particles which may be a disadvantage since DCs (natural APCs) are not spherical and for that reason have a bigger contact area with T cells than aAPCs.

The use of aAPCs has become a successful tool in immunology and clinical applications, especially the ones made with biomaterials which generate anti-tumor immune responses both in vitro and in vivo. Some advantages of these systems are the fact that they are not time-consuming in manufacture or preparation and they do not need expensive cell-culture strategies.[50], [52]

In these type of systems, the three signals that are needed for T cell activation are mimetized usually using agonist molecules. For example, signal 1 is provided by TCR agonists, such as recombinant peptide-MHC complexes or antibodies that are directed to CD3. Signal 2 is co-

stimulation and it is provided by co-stimulatory agonists, for example anti-CD28 monoclonal antibodies. Cytokines are responsible for signal 3 and they play an important role in T cell expansion and differentiation. IL-2 is the cytokine most commonly used to guarantee signal 3 since it is the best known for the survival of $CD8^+$ T cells. However, there are other cytokines that can promote, even better, expansion and differentiation of T cells. In these group of cytokines are included IL-7, IL-15 and IL-21.[53], [54]

Currently, there are some clinical trials where aAPCs have been tested. For example, anti-CD3/anti-CD28-coated magnetic beads, a type of aAPCs, have been applied in Phase I clinical trials. These aAPCs were tested in patients with metastatic breast cancer, chronic myeloid leukemia and carcinomas. These trials showed, in some cases, tumor regression or even complete remission but also showed a risk of developing graft versus host disease or non-tumor-specific cytotoxicity [50].

Silica microspheres containing peptide-MHC I complexes or tumor cell membranes were tested first in mice where they induced regression of tumors in combination with chemotherapeutic agent cyclophosphamide. In Phase I and II clinical trials these aAPCs were tested in patients with melanoma and renal cell carcinoma. It was observed a variable success where partial responses were induced, in some cases [50].

1.3.1 Cell Based aAPCs

There are aAPCs based on xenogenic or allogenic cells genetically modified, such as *Drosophila* cells, murine fibroblasts and human erythroleukemia cells (K562) [55]. These cellular-aAPCs present several advantages, since these are stable cell lines that can be easily handled, stored for long periods and easily obtained. Cells used in this system are also better defined than DCs, which allows a more controlled system. The biggest disadvantage is related with the allogenic nature of cells.[50], [55]

To be used as aAPCs, cells are engineered by transduction with retroviral or lentiviral vectors. Transduction allows introduction of necessary molecules needed for the immune synapse formation, such as TCR, costimulatory and adhesion molecules. Besides these, cytokines can be introduced in culture medium or produced by cellular-aAPCs.[55], [56]

1.3.2 Synthetic/Acellular aAPCs

Acellular aAPCs are relatively easy to prepare, provide a more stringent control when compared to cellular aAPCs and can be nonspecific or antigen-specific because they can activate T cells either presenting T cell activating antibodies, such as anti-CD3, or presenting specific peptide-MHC complexes. Most of aAPCs approaches use MHC stimulation for $CD8^+$ T cells activation, since these cells have antigen specific tumor cell lysis ability. There are some properties of aAPCs that will influence T cell activation. Size, shape, distribution of ligands in the

surface and ligand mobilities are some of those properties. Therefore, many types of aAPCs have been developed in order to understand which ones are better to mimic DCs.[50], [52]

Currently there are many systems of synthetic aAPCs being developed. These systems are based on synthetic and biomimetic biomaterials and they present applications in immunotherapy either *ex vivo* and *in vivo*.[53]

Liposomes, RAFTsomes and microdomain liposomes are lipid based aAPCs which are particles with a lipid bilayer and a fluid membrane. Liposomes containing MHC were developed by Prakken et al., and were able to activate $CD4^+$ T cells in vitro [57]. Ding et al. developed the RAFTsomes which are liposomes containing pre-clustered peptide-MHC complexes on membrane microdomains. These ones were capable of promoting $CD4^+$ T cell proliferation [58]. Giannoni et al. described other systems based on artificial membrane bilayers containing T cell ligands membrane microdomains [59]. Since liposomes are poorly stable, some scaffolds started to be used. These scaffolds have as main function to support the lipid bilayers and improve the advantages of using these systems as aAPCs. Lipid bilayers were constructed on Poly(lactic-co-glycolic acid) (PLGA), silica or core of hydrogel.[53]

Polymeric aAPCs are another type of aAPCs that are made from polymers. The most common used polymers PLGA, sepharose or polystyrene beads. The polymeric particles present immunomodulatory compounds (anti-CD3, anti-CD28, peptide-MHC complexes) on the surface and can release gradually the IL-2 or other soluble molecules that were encapsulated inside. In this case, the optimal size of microbeads was between 4 to 5 μm. [50]

Since natural APCs are not spherical and the shape is a very important parameter for T cell activation, there were also designed non-spherical microparticles. Ellipsoid PLGA microparticles made using film-stretching technologies are one of these examples. Like this, it is possible to better mimic what happens *in vivo*. [50], [53]

Carbon nanotube bundles, other type of aAPC, are functionalized nanotubes coated with anti-CD3 that seem to be grouped into large aggregates with a high surface area which contribute to a better T cell activation. [53]

Nanoworms are aAPCs that combine two important aspects, membrane fluidity and shape, to access better results. For this, semi-flexible filamentous polymers are used to form the nanoworms that, then, can be decorated with antigen presenting and co-stimulatory molecules.[50]

There are also aAPCs that contain magnetic parts. Levine et al. describe magnetic beads linked to anti-CD3 and anti-CD28 antibodies. These beads were applied in clinical trials as a potential treatment for different types of cancers. [53]

1.3.3 Polyelectrolyte Microcapsules (PEMs)

Polyelectrolyte Microcapsules (PEMs) are a type of synthetic aAPCs that were produced at Sebastian Springer's laboratory in Jacobs University at Bremen, Germany and their performance was tested in the context of this thesis.

PEMs are carbon microcapsules that were produced using calcium carbonate particles with 5-6 µm of size. The calcium carbonate particles were coated with two layers of PAH (Poly(allylamine hydrochloride)) and PAA (Poly(acrylic acid) sodium salt) in alteration. After that, the –COOH groups on the microcapsules were activated with EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimid) and Sulfo-NHS (Sulfo N-Hydroxysuccinimide) and the streptavidin molecules were added in order to coat the PEMs [60].

1.4 Aim of Thesis

Cancer vaccines are one type of immunotherapy, especially vaccines of DCs. More recently, aAPCs have been developed in order to increase the safety and efficacy of treatment. In the last case, aAPCs are used to expand and activate T cells against specific tumor antigens.

The main aim of this thesis is to test a new generation of aAPCs and to study their potential to activate T cells *in vitro* (Figure 1.5). aAPCs tested in this thesis were microcapsules made and assembled by researchers at the Jacobs University and named PEMs.

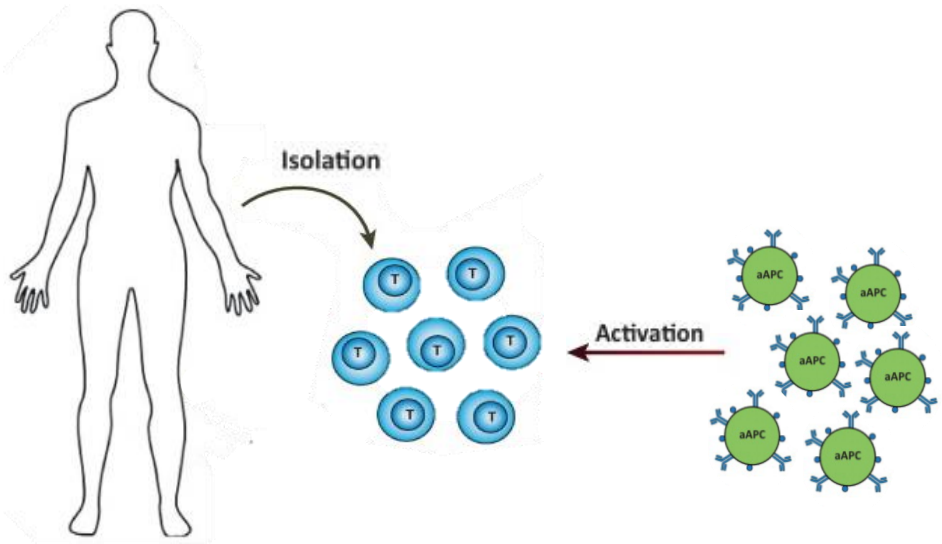


Figure 1.5 - One strategy of cancer immunotherapy. *Activation of T cells isolated from patients can be induced by aAPCs. Adapted from* [50].

PEMs microcapsules have a carbonate core and are coated with streptavidin molecules. In addition to PEMs, three types of HLA-A02 molecules biotinylated were used in this thesis: HLA-A02 molecules with no peptide (empty), with NV9 peptide, which is a peptide derived from cytomegalovirus (CMV), and also HLA-A02 molecules with IV9 peptide, a peptide derived from proteins of the human immunodeficiency virus (HIV). Since HLA-A02 molecules were biotinylated and PEMs were coated with streptavidin it was possible to bind the two components by a streptavidin-biotin reaction.

HLA-A02 molecules were used as a model because this allele is one of the most prevalent in the population. The CMV peptide was chosen as the peptide of interest since 60% to 90% of the adults have suffered a CMV infection at some point in their life [61]. The HIV peptide was

chosen as peptide of control since the cells used in the tests were from healthy donors tested for HIV.

In order to test the capacity of PEMs to activate T cells, co-cultures of $CD8^+$ T cells and PEMs were made. Since all of the technical system was optimized with HLA-A02 molecules, the T cells used in co-cultures had to be T cells isolated from HLA-A02 positive donors. The first step was to perform a screening of HLA-A02 positive donors by flow cytometry. After that, T cells were isolated from positive donors by a density gradient in Biocoll. To test if PEMs were capable to activate T cells, the co-cultures were maintained for different time points. To test the activation of T cells the expression of IFN-γ was evaluated at transcriptional and protein level.

Since PEMs technology use CMV peptide as peptide of interest, only the $CD8^+$ T cells from donors previously infected with CMV and with lymphocytes responsive to CMV will produce IFN-γ in response to the stimulus. Figure 1.6 represent a schematic resume of the principal steps and methods used in this thesis to achieve the aim.

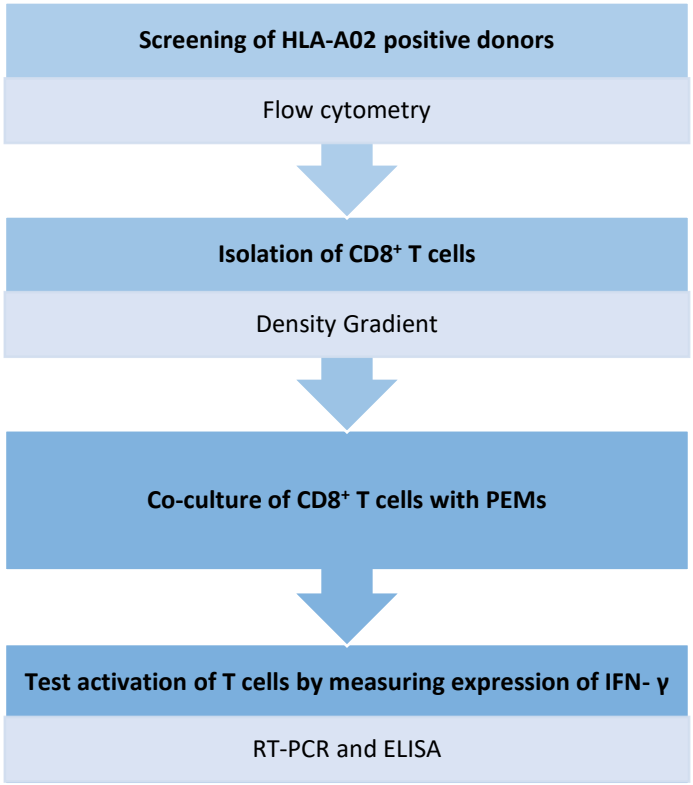


Figure 1.6 - Schematic resume of the aim of the thesis. *Principal steps and respective methods used. First step consists in screening of HLA-A02 positive donors by flow cytometry. Second step consists in the isolation of $CD8^+$ T cells by density gradient and then co-culture of the isolated cells with PEMs. Final step was testing the activation of T cells measuring the expression of IFN-γ by RT-PCR and ELISA.*

2 Materials and Methods

2.1 Flow cytometry

Flow cytometry is a sophisticated and powerful tool that allows a rapid analysis of multiple individual parameters of single cells, or other particles such as nuclei, microorganisms, chromosome preparations and latex beads. This technique allows to measure the optical and fluorescence characteristics of a suspension of particles [62], [63].

Flow cytometers are composed by three principal components: fluidics, optics and electronics. The fluidic system introduces and aligns the particles in a continuous flow in direction to the laser beam allowing a single cell or particle to go through the light laser. The optical system is divided into excitation components and collection components. The first one is responsible for focusing the light source on the particles and the collection components capture the dispersion and emitted fluorescent light by the fluorophores that are excited by the laser beam. This information is transmitted to the electronic system that converts the signal into digital data.[64]

In flow cytometers, the particles pass one by one through a laser beam and the scattered lights at different angles (related with structure and morphology of the cells) and fluorescence emissions are measured. Size, granularity, which are related with the internal complexity of the cell, and fluorescent features are physical properties that are used to characterize and differentiate cell populations.[65]

The forward angle light scatter (forward-scatter) is related with the size of the cells and the complexity of the cells is represented by the right angle scatter and it is known as side-scatter.[63]

The fluorescent emissions which are measured can be derived from natural or chemical molecules. The last ones are used to stain some components that do not have natural fluorescence.[64]

Fluorescent compounds absorb light energy over a range of specific wavelengths. This absorption allows an electron to move into its excited state, as it passes from its fundamental level of energy to a higher energy level. Once the excited electron quickly returns to its ground state the excess of energy is released as a photon of light, being this release of energy called fluorescence. [62]

The fluorescent dyes can bind to different cellular components and antibodies conjugated to fluorescent dyes can bind with more specificity to the cellular components, such as proteins or cell membranes. The amount of fluorescence is proportional to the amount of fluorescent probe that is bound to a specific cell or component.[64]

For the work of this thesis, the Flow Cytometer that was used was an Attune Acoustic Focusing Cytometer (Applied Biosystems) which is constituted by two lasers: a blue laser (488 nm) and a red laser (638 nm). These two lasers allow the use and detection of up to six different fluorescences: four detected by the blue laser (BL1: green, BL2: orange, BL3: red and BL4: red) and the other two detected by the red laser (RL1: light red, RL2: dark red). In all experiments performed, at least $1x10^4$ events were acquired using the Attune Cytometric Software (version

2.1) and all the data analysis was made using FlowJo (version 10) and GraphPad Prism (version 6).

2.1.1 Screening of HLA-A02 positive donors by flow cytometry

Since the PEMs used in this experimental setup were assembled exclusively with MHC I molecules of the HLA-A02 allele, only $CD8^+$ T cells from HLA-A02 donors were selected.

T cells used in the different assays were isolated from buffy-coats which are leuco-platelet concentrates. Buffy-coats were provided by Instituto Português do Sangue e da Transplantação (IPST), and all blood collection, separation of components and ethical procedures were performed by this institute. Each buffy-coat contains approximately 60 mL of blood and was obtained from a single healthy donor. Also in IPST, donated blood was screened in order to guarantee that the donors were healthy. An analytic imuno-serologic and molecular biologic screening for hepatitis B and hepatitis C virus, human immunodeficiency virus (HIV1/2), human T-lymphotropic virus (HTLV) and *Treponema pallidum* bacteria, which is syphilis agent, is performed prior to buffy coat receival.

Since all PEMs technology was optimized for cells from HLA-A02 donors, the first step performed was the screening of HLA-A02 positive donors by flow cytometry. To test if donors were HLA-A02 positive an aliquot of 100 µL of the leuco-platelet concentrate was placed into two tubes of 2 mL (100 µL in each tube) to perform a red blood cell lysis step at the same time that cells were stained for flow cytometry. In one of the tubes, 3 µL of PE Mouse anti-human HLA-A02 Clone BB7.2 (BD Pharmingen™) was added and mixed thoroughly. The tubes were then incubated 30 minutes in the dark at room temperature. After incubation, 2 mL of room temperature Red Blood Cells Lysis Buffer 1x (see composition on Appendix I) were added in each tube. Tubes were pulsed in vortex and incubated at room temperature in the dark for 15 minutes. After lysis, the tubes were centrifuged at 500 x *g* for 5 minutes at room temperature. The supernatant was decanted and cells resuspended in 2 mL of phosphate buffered saline (PBS) (see composition on Appendix I) and centrifuged again at 500 x *g* for 5 minutes at room temperature. Finally, the supernatant was decanted and cell pellet resuspended in PBS. The samples were then analyzed by flow cytometry. An unstained control was used in parallel. The strategy used to analyze if donors were HLA-A02 positive is demonstrated in the Figure 2.1.

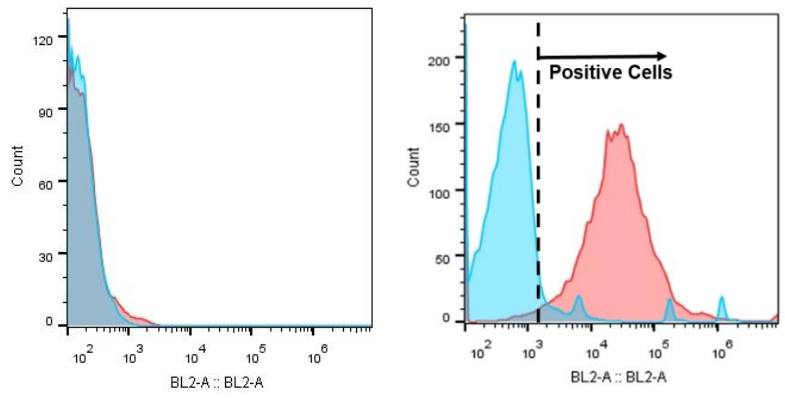


Figure 2.1 - Strategy used to analyze if the donors tested were HLA-A02 positive or not. *After flow cytometry acquisitions, a histogram graph of fluorescence channel vs count was generated and if the peak of the cells (red) was equal to the peak of unstained cells (blue) the donor was HLA-A02 negative, as shown on the right side. Otherwise, if the peak (red) was displaced relatively to the peak of unstained cells (blue) the donor was HLA-A02 positive, as shown on the left side.*

2.2 Isolation of human peripheral blood mononuclear cells from buffy coats by density gradient

In order to isolate $CD8^+$ T cells from buffy-coats of HLA-A02 positive individuals, peripheral blood mononuclear cells (PBMCs) were separated from the other components, namely erythrocytes, granulocytes and blood plasma. The separation of the different components was made by density gradient centrifugation using Biocoll (Biochrom AG) separation solution (with density of 1.077g/mL), which is denser than the lymphocytes and monocytes and less dense than the erythrocytes and granulocytes.

The Biocoll separation solution was pre-warmed at room temperature and added to two 50 mL tubes (12,5 mL/tube). Then, 25 mL of the buffy coat was slowly added to each tube in order to not disturb the Biocoll gradient. The tubes were centrifuged at 1300 x *g*, for 30 minutes, at room temperature, without braking. After centrifugation, the mononuclear cells rings were collected and added to a new tube of 50 mL and resuspended with PBS until 50 mL. The samples were centrifuged at 900 x *g*, for 10 minutes at room temperature for platelet removal. The supernatant was removed and the pellet was resuspended in PBS. Then PBS was added until 50 mL and a sample of PBMCs was collected for counting using a Neubauer chamber. The sample was centrifuged again at 900 x *g*, for 10 minutes at room temperature. After this, a purified fraction consisting of PBMCs was obtained for isolation of $CD8^+$ T cells.

2.3 Immunomagnetic isolation of $CD8^+$ T cells from human peripheral blood mononuclear cells

$CD8^+$ T cells were separated from PBMCs, which were previously isolated from buffy coats. To isolate $CD8^+$ T cells, PBMCs were resuspended in 80 µL per each 10^7 cells of cold Beads Buffer (see composition on Appendix I) and in 20 µL of human CD8 magnetic microbeads per each $30x10^6$ cells. After this step, the cell suspension was incubated at 4ºC for 15 to 30 minutes. Then, 2 mL of cold beads buffer was added per each 10^7 cells and this cell suspension was centrifuged for 10 minutes at 300 x *g*. After centrifugation, the pellet was resuspended in 500 µL of cold beads buffer per each 10^7 cells and the suspension was filtered using a BD Falcon cell strainer (100 µm) in order to remove aggregates. Cell suspension was then added to a Miltenyi Biotec magnetic separation LS column previously prepared, placed in a support with a magnetic field (MidiMACS Separator (Miltenyi Biotec)) and washed with 3 mL of cold beads buffer. Inside the column, due to the magnetic field, the $CD8^+$ fraction (labeled cells) stay immobilized in the

column while $CD8^-$ fraction (unlabeled cells) were eluted from the column to a 15 mL tube. Then, a wash step is performed by rinsing the column three times using 3 mL of cold beads buffer. In the end, to collect the $CD8^+$ fraction, the column was removed from the magnetic field, 5 mL of cold beads buffer was added and the cells were eluted to a 15 mL tube inserting the plunger into the column. In order to count the cells in the Neubauer chamber, a sample of each fraction was collected. The samples were then centrifuged for 10 minutes at 400 x *g*. $CD8^+$ T cells were then cultured as described in the next section.

2.4 Culture of $CD8^+$ T cells

$CD8^+$ T cells were cultured in T-75 flasks (Sarstedt) using Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco) supplemented with 10% (v/v) of Fetal bovine serum (FBS) (Gibco), 2mM L-glutamine (Gibco), 100U/mL of penicillin (Gibco), 100 μg/mL of streptomycin (Gibco), 1mM sodium pyruvate (Gibco) and 1% (v/v) MEM non-essential amino acids (Gibco). Cells were maintained in culture until they were not needed for any assays. Medium was changed every 4-6 days.

2.5 Efficacy of isolation method

After isolating $CD8^+$ cells from the buffy coats, the efficacy of the method used for isolation was tested by flow cytometry. An aliquot of isolated cells was centrifuged at 400 x *g* for 5 minutes and after supernatant discard the pellet was resuspended in 400 μL of PBS. Cell suspension was distributed on 4 tubes (100 μL in each tube). Cells were stained using anti-CD3 Allophycocyanin (APC) Clone MEM-57 (ImmunoTools) and anti-CD8 Peridinin chlorophyll protein complex (PerCp) Clone UCHT-4 (ImmunoTools). Unstained controls were performed in parallel. After these steps, the tubes were incubated for 15 minutes, at room temperature in the dark. Cells were washed and centrifuged at 400 x *g* for 5 minutes and the supernatant discarded. Cells were then resuspended in 1 mL of PBS and analyzed by flow cytometry.

The tubes with unstained cells, cells marked with anti-CD3 APC alone and cells marked with anti-CD8 PerCp alone were used for compensation purposes. The percentage of $CD8^+$ T lymphocytes isolated from the buffy coat could then be determined. To do this analysis, it is important to quantify the cells that were marked with the two antibodies which were the $CD8^+$ cells. Double marked cells appear in the quadrant Q2 (Figure 2.2).

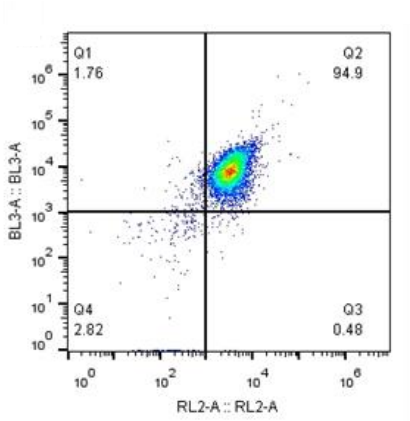


Figure 2.2 - Gating strategy to evaluate the efficacy of gradient density isolation method. *Cells isolated were marked with anti-CD3 APC and anti-CD8 PerCp. Double marked cells correspond to CD8⁺ T cells in Q2 quadrant*

2.6 Assembly of PEMs

PEMs, as mentioned before, were synthetized by our collaborators at Jacob's University Bremen. Besides PEMs, which are coated with streptavidin, also biotinylated HLA-A02 molecules were synthetized there. Our collaborators produced three types of biotinylated HLA-A02 molecules: HLA-A02 molecules empty (without any peptide); HLA-A02 molecules complexed with a peptide of cytomegalovirus (CMV) and HLA-A02 molecules complexed with a peptide of human immunodeficiency virus (HIV). The peptide of CMV is designed NV9 (sequence NLVPMVATV) and it is the peptide of interest for providing T cell stimulation. The peptide of HIV is designed IV9 (sequence ILKEPVHGV) and corresponds to an irrelevant peptide control in the experiments.

Firstly, aAPCs are required to be assembled. This is achieved by joining streptavidin-coated PEMs to biotinylated HLA-A02 molecules, promoting a streptavidin-biotin reaction. This reaction is performed by incubating the PEMs with HLA-A02 molecules at 4ºC for 30 minutes. Following our collaborators advice, approximatly 200000 capsules would bind 1 ng of HLA protein and all experiments were made with this guidance.

2.7 Co-culture of CD8⁺ T cells with PEMs

After conjugating PEMs with HLA-A02 molecules, these complexes were incubated with cultured CD8⁺ T cells isolated from healthy donors as described above. A microcapsule to cell ratio of 1:1 was used for all experiments. Co-cultures were maintained in RPMI complete medium with 10% FBS for up to 72 hours. Co-cultures were made in a 96-well plate round bottom for six conditions. Replicates were performed for each condition. 50000 CD8⁺ T cells were seeded in each well. CD8⁺ T cells alone were used as negative control. Phorbol 12-myristate 13-acetate (PMA) and Ionomycin (Ion) were used as positive controls to stimulate and activate CD8⁺ T cells.

Experimental conditions included: (1) empty PEMs without HLA-A2 molecules; (2) PEMs complexed with empty HLA-A02 molecules; (3) PEMs complexed with NV9-HLA-A02 molecules

and (4) PEMs complexed with IV9-HLA-A02 molecules. Table 2.1 resume all conditions tested in duplicate for each donor.

Table 2.1 – Conditions tested in the experiments.

Conditions Tested (in duplicate for each donor)
$CD8^+$ T cells
$CD8^+$ T cells + PMA + Ion
$CD8^+$ T cells + PEMs
$CD8^+$ T cells + PEMs + HLA-A02 empty
$CD8^+$ T cells + PEMs + HLA-A02 + NV9
$CD8^+$ T cells + PEMs + HLA-A02 + IV9

2.8 Real time PCR

Real time polymerase chain reaction (real time PCR), also known as quantitative PCR (qPCR) is a powerful and sensitive technology for gene expression analysis. In traditional polymerase chain reaction (PCR), deoxyribonucleic acid (DNA) or complementary deoxyribonucleic acid (cDNA) are used as templates to amplify specific sequences. In this case, detection and quantification can only be made in the end of the reaction and analysis usually involves gel electrophoresis and imaging analysis. However, in qPCR, quantification is made at each cycle since with this technique it is possible to control reactions at exponential amplification. For quantification, fluorescent dyes are used and fluorescent signals increase proportionally to the number of PCR product molecules making possible the determination of initial concentration of DNA or ribonucleic acid (RNA). There are different types of fluorescent dyes that can be used: double-strand DNA, PCR primers or probes linked to fluorescent dyes. The data collected from the exponential phase of real time PCR is used for quantification and in this phase the instrument calculates the threshold and threshold cycle (Ct). Ct is essential for absolute or relative quantification and corresponds to the number of the cycle in which the threshold was reached.[66]

Reactions of real time PCR are usually run for 40 cycles and in each cycle there are three principal steps. The first step corresponds to denaturation, where double-stranded DNA is melted due to the increase of temperature and single-stranded DNA is formed. The second step is named annealing and here an optimal temperature is used to promote the hybridizing of complementary sequences to the single stranded DNA formed in the first step. The last step is elongation, consisting on primer extension. In this step, the temperature is set around 72ºC, which is the optimal temperature for the DNA polymerase activity.[67]

In the work developed in this thesis, a two-step qRT-PCR was performed. It consists of using a reverse transcriptase enzyme to perform a reverse transcription of total RNA into cDNA. After this step, part of cDNA is placed in the tube where real-time PCR reaction will take place.

Regarding detection chemistry, there are two types that have been developed: TaqMan chemistry and SYBR Green I dye chemistry. In the context of this thesis, TaqMan was used, which is also known as fluorogenic 5' nuclease chemistry. This chemistry is based on two phenomena: the first is the capacity that 5' nuclease domain has to break down, downstream of DNA synthesis, the DNA that is bound to template and the second is the fluorescence resonance energy transfer (FRET) phenomenon. In this phenomenon, fluorescent emissions of one dye (reporter) are suppressed by the nearest presence of another dye (quencher). So, in TaqMan chemistry, exclusive TaqMan probes are used. Probes are constituted by a reporter, bound to the 5' end, and a quencher, bound to the 3' end, that allows the FRET phenomenon to occur, since they have affinity to each other. In the beginning of the reaction the probe is intact, these are the reporter and the quencher that are near each other because their affinity and the FRET phenomenon is occurring. When PCR reaction begins, both primers and probe anneal to the target sequence of the template. DNA polymerase start their activity and extend the primer. When DNA polymerase achieves the probe cleaves it with her 5' nuclease activity. The cleavage separates the quencher from the reporter and its fluorescence is restored. During the process many probes are cleavaged and the fluorescent intensity starts to increase proportionally to the amount of amplicon formed [68]. In order to quantify the relative gene expression using the data collected by real-time PCR experiments, the $2^{-\Delta\Delta Ct}$ method, developed by Livak and Schmittgen [69], also known as comparative threshold method was used. With this method, it is possible to calculate the relative expression of a target gene relatively to a reference control. Reference control is given by the expression of an endogenous housekeeping gene. An ideal housekeeping gene is a gene that presents a constant expression independently of experimental conditions, such as the tissue analyzed, cell type and stage of development. Two of the most common genes used as control genes and the ones that were used in the experiments of this thesis are *ACTB* gene, that codes β-actin (a cytoskeletal protein), and *GADPH* which encodes for glyceraldehyde-3-phosphate dehydrogenase, a glycolytic enzyme. Using the mathematical method mentioned before, it was possible to calculate the amount of target gene in the sample. This amount normalized to endogenous control and relative to the normalized calibrator is given by $2^{-\Delta\Delta Ct}$, where Ct indicates the cycle number in which the fluorescent signal reaches the threshold; ΔCt indicates the variation between the Ct of the target gene and the Ct of the housekeeping gene and ΔΔCt corresponds to the variation of ΔCt of the sample of interest and the ΔCt of the calibrator [69], [70].

2.8.1 Analysis of genetic expression of IFN-γ in the cells recovery from co-cultures experiments, by RT-PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) protocol was performed to analyze the genetic expression of IFN-γ in the cells recovered from the co-cultures. Total RNA was extracted from T cells using the GenEluate™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich)[71]. All steps were carried at 4ºC. Cells were lysed using the lysis buffer (1% of 2-

Mercaptoethanol (Sigma) per 1 mL of lysis solution. Approximately 5 x 10^6 cells were used per lysis. Total cell lysates were passed into a filtration column and centrifuge at 12000 x *g*, for 2 minutes. The filtration column was then discarded and 250 μL of 70% of ethanol was added to the filtered lysate, pipetting thoroughly. The lysate/ethanol mixture was placed into a binding column for RNA retrieval and centrifuged at 1200 x g for 2 minutes. The flow-through liquid was discarded. 250 μL of wash solution 1 was added to the column and centrifuged at 1200 x *g* for 2 minutes. The flow-through liquid was discarded and the DNA digestion was performed. For this, DNase I (Sigma) was prepared mixing 10 μL of DNase I with 70 μL of digest buffer by inversion. The 80 μL of the mixture prepared was added directly onto the filter in the binding column and the tubes were incubated for 15 minutes at room temperature. After incubation period, 250 μL of wash solution 1 was added into the column and centrifuge at 1200 x *g* for 2 minutes. The flow-through liquid was discarded and 500 μL of wash solution 2 was pipetted into the column, centrifuged at 1200 x *g* for 2 minutes and the flow-through liquid discarded again. A second 500 μL of wash solution 2 was added into the column, centrifuged at 1200 x *g* for 2 minutes and the flow-through liquid discarded. After that another centrifugation at 1200 x *g* for 2 minutes was performed and then the binding column was placed into a fresh 2 mL collection tube. In order to elute the RNA, 55 μL of elution solution was added into the binding column and centrifuged at 1200 x *g* for 2 minutes. The flow-through liquid was pipetted again to the column and centrifuged at 1200 x *g* for 2 minutes. At this point the purified RNA was recovered from the flow-through liquid.

The second step of RT-PCR protocol was conversion of RNA to cDNA, which was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). In this conversion it was used random primers which can hybridize with several regions of RNA allowed transcription by reverse transcriptase enzyme. To do the conversion, 50 μL of the purified extracted RNA and 50 μL of the conversion mix were added to a PCR tube. The content of conversion mix and respective amounts are described in the following table.

Table 2.2 – Components of conversion mix and respective amounts (μL)

Components	**Amounts (μL)**
Buffer (10x)	10
Random primers (10x)	10
dNTP's (100 mM)	4
Reverse transcriptase enzyme	5
RNase free H_2O	21

The cDNA synthesis was then performed on a Programmable Thermal Controller PTC-100™ (MJ Research, Inc.) thermocycler according to the program mentioned in the table. After conversion the samples were stored at -20ºC until real-time PCR was performed.

Table 2.3 – Settings of the used program to cDNA conversion

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (ºC)	25	37	85	4
Time	10 min	120 min	5 min	∞

For RT-PCR experiments, the samples were prepared in RT-PCR tubes (Simport) to a final volume of 10 μL. Each sample was prepared in duplicate and adding 2 μL of 1:4 diluted probe, 2 μL of cDNA, 1 μL of RNase free H_2O and 5 μL of TaqMan Fast Universal PCR Master Mix 2x (Applied Biosystems) per tube. The probe of interest was IFN-γ (Hs00174143_m1, Applied Biosystems) and the endogenous control were GADPH (Hs03929097_g1, Applied Biosystems) and β-actin (Hs99999903_m1, Applied Biosystems). Reactions were run on a Rotor-Gene 6000 Sereies (Corbett) according to table 2.4.

Table 2.4 – Settings of the used program to RT-PCR assay

	Step 1 (1 cycle)	**Step 2 (40-50 cycles)**	
Temperature (ºC)	95	95	60
Time (seconds)	20	3	30

C_T was determined by using Rotor-Gene 6000 Sereis software (version 1.7). Relative messenger ribonucleic acid (mRNA) levels were calculated with $2^{-\Delta CT}$ x 1000 equation and normalized against arithmetic means of the expression of the endogenous genes (GADPH and β-actin). The equation allows the calculation of mRNA molecules of each gene of interest per 1000 molecules of endogenous controls. Results were analyzed using Microsoft Office Excel and GraphPad Prism (version 6).

2.9 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a colorimetric technique that allows qualitative or quantitative determinations of antigens or antibodies and it is based on the concept that an antigen binds to its specific antibody. ELISA is used as a diagnostic tool and also in biomedical research [72]. With this assay, it is possible to detect small amounts of antigens (proteins, peptides, hormones) or antibodies in a fluid sample. Typically, the first step of ELISA consists on the immobilization of an antigen on wells of a 96 well microplate. After that, test antibodies specific for the antigen are added followed by the addition of secondary antibodies conjugated with enzyme which are against test antibodies. Last step consists of the addition of a chromogenic substrate for the enzyme which will give color, fluorescent or luminescent products

that allows determination of reagents concentrations. Colorimetric reading provides quantitative or qualitative measures. Most commonly used enzymes in ELISA are horseradish peroxidase (HRP) and alkaline phosphatase (ALP) and the most commonly used chromogenic substrate is 3,3',5,5'-tetramethylbenzidine (TMB). There are various types of ELISAs due to variations that are made to the procedure. In this thesis it was used a variant called capture ELISA. This type of ELISA is used to detect a specific antigen in a sample and for that a capture antibody specific for the antigen is immobilized on the plate. After that the sample containing the antigen of interest is added to each well and the specific antigen will bind to the immobilized antibody. At last, another antibody specific for the antigen is added (the binding antibody). This latter antibody allows detection of the antigen after the HRP enzyme and the chromogenic substrate is added [73]–[75].

In the ELISA's performed for the work of this thesis, a biotin-streptavidin system was used, since the binding antibody used is biotinylated and HRP enzyme is conjugated with streptavidin which amplifies the signal since the amount of enzyme bound is increased. TMB was used as chromogenic substrate. When HRP enzyme reacts with TMB, a blue color appears. After the addition of an acidic solution, blue color turns yellow that can be detected on a spectrophotometer plate reader at 450 nm wavelength. SpectraMax 190 Microplate Reder (Molecular Devices) and SoftMax Pro were used to read the plates and collect the data, respectively. Collected data were analyzed using Microsoft Office Excel and GraphPad Prim 6.

2.9.1 Capture ELISA for assessment of IFN-γ in co-culture supernatants

In order to do a absolute quantification of IFN-γ in co-cultures supernatants, an ELISA kit (Immunotools) was used following manufacturer's instructions. To do ELISA technique, the wells of a high binding 96-well plate (Costar) were coated with 50 μL of capture antibody diluted 1:100 in PBS for ELISA. The plate was incubated at 4ºC, overnight. After incubation period, the captured antibody was removed and 200 μL of blocking buffer (see compositon on Appendix I) was added to each well. At this point, the plate was incubated for 1 hour at room temperature. To do a standard curve, the standards were prepared with a serial dilution of 1:2 from 4000 pg/mL to 0 pg/mL. To do the dilutions it was used blocking buffer. After 1 hour incubation, the blocking buffer was removed. 50 μL of each standard and each sample were added, in duplicate, in their respective wells. The plate was then incubated for 2 hours at room temperature. Afterwards, the plate was washed five times with 200 μL of washing buffer (see composition on Appendix I) for each well at each time. Biotinylated antibody was diluted 1:100 in blocking buffer, 50 μL of this antibody was added to each well and the plate was incubated 2 hours at room temperature. Plate was again washed five times with 200 μL of washing buffer for each well and 50 μL of Poly-HRP-Streptavidin-HS enzyme previously diluted 1:1000 were added to each well. After 30 minutes incubation at room temperature, the plate was washed again with the same conditions and 50 μL of TMB substrate was added at each well. The plate was incubated in the dark at room temperature until optical color development. Finally, the reaction was stopped by addition of 25 μL of 4M HCl and the optical density was read at 450 nm.

2.10 Experiment to test the link between PEMs and $CD8^+$ cells

In order to test if PEMs can bind to $CD8^+$ cells, these two were co-cultured for 24 hours in BD Falcon Conical Tubes and the results were analyzed by flow cytometry.

Experimental conditions included the co-culture of $CD8^+$ T cells with: (1) PEMs; (2) PEMs containing empty HLA-A02 molecules; (3) PEMs containing NV9-HLA-A02 molecules and (4) PEMs containing IV9-HLA-A02 molecules. Unstimulated $CD8^+$ T cells were used as control.

For flow cytometry analysis, after PEMs preparation (as described in section 2.6), biotinylated fluorescein isothiocyanate (FITC) was added in each tube in order to compete with the biotinylated HLA-A02 molecules for the streptavidin binding site. After assembly, PEMs were cultured with $CD8^+$ cells using the different conditions for 24 hours. At the defined time point, cells were stained with an anti-CD8 phycoerythrin (PE)-conjugated antibody. Samples were analyzed by flow cytometry, using a proper gating strategy.

3 Results and Discussion

The work developed for this thesis had as main objective to test the capacity of PEMs to activate T cells. These aAPCs were synthesized with the aim of mimicking the role that dendritic cells have *in vivo.* Previously, PEMs were tested using murine lymphocytes, and the activation or response was measured by the secretion of IFN-γ. The assays developed within this thesis aimed to test the PEMs ability to activate human lymphocytes.

3.1 Screening of HLA-A02 positive donors

Since all of PEMs technology was developed for HLA-A02 positive cells, the first step was to isolate $CD8^+$ T cells from HLA-A02 donors. For this purpose, the buffy coats provided by IPST were tested in order to find HLA-A02 positive donors. These tests were done by flow cytometry as described above. The graphs below (Figure 3.1) correspond to the results of the buffy coats tested. The strategy used to analyze the results is described in section 2.1.1.

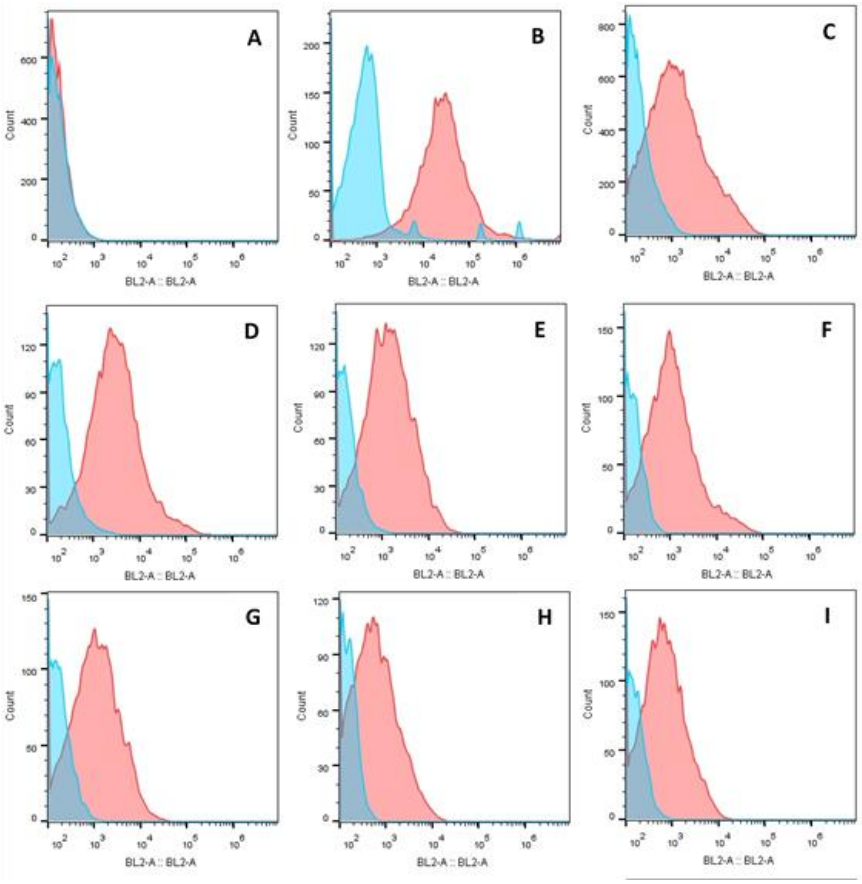


Figure 3.1 - Screening of HLA-A02 donor by flow cytometry. *On the histograms the yy axis represent the number of events and the xx axis represents the fluorescence intensity on the BL2 channel. In blue are represented the unstained cells and in red the cells stained with HLA-A02 PE. A – Two histogramss (blue and red) are overlapped, representing a HLA-A02 negative donor. B,C,D,E,F,G,H,I – The two histograms that are not overlapped being the red peak (stained cells) moved to the right. These represent HLA-A02 positive donors.*

The Figure 3.1A corresponds to a HLA-A02 negative donor, since the two histograms (blue - unstained cells; and red - marked cells) are overlapping, and all other graphs (Figures 3.1B to 3.1I) correspond to HLA-A02 positive donors. There were many other HLA-A02 negative donors that were tested but since the buffy coats from that donors were not used those results were omitted. In total 40 buffy coats were tested, being 70% from male donors and 30% from female donors. The buffy coats were from donors with age between 18 and 65 years. The cells isolated from the buffy coat of donor A were used as a negative control.

After testing whether the donors were HLA-A02 positive or not, $CD8^+$ T cells were isolated by the density gradient method previously described.

3.2 Efficacy of $CD8^+$ T cells isolation

In order to check the effectiveness of the isolation of $CD8^+$ cells from buffy coats, flow cytometry was used to see if cells isolated were in fact $CD8^+$ T cells. In this analysis, antibodies recognizing specific surface markers of $CD8^+$ cells, such as CD3 and CD8, were used. The protocol analysis strategy used were described in section 2.5.

This analysis was only done on cells isolated from the first six donors to prove the effectiveness of the method used for isolation.

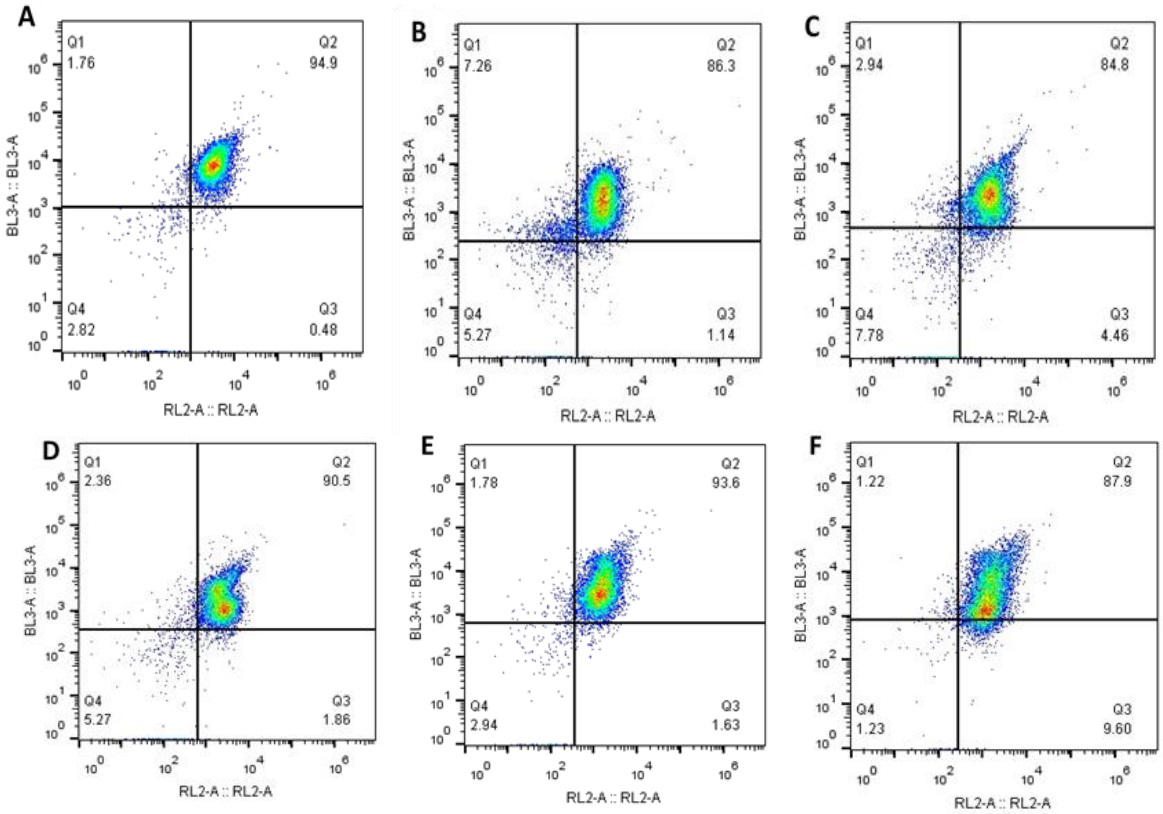


Figure 3.2 - Percentage of $CD8^+$ T cells isolated from each from donors A,B,C,D,E and F. *$CD8^+$ T cells appear on Q2 quadrant since cells isolated were stained with two markers (CD3 APC and CD8 PerCp) to distinguish $CD8^+$ T cells. On Q2 quadrant appear the double stained cells, so the $CD8^+$ T cells. A – Cells isolated from donor A, being 94.9% of the cells isolated $CD8^+$ T cells. B – Cells isolated from donor B, being 86.3% of the cells $CD8^+$ T cells. C – Cells isolated from donor C. 84.8% of isolated cells were $CD8^+$*

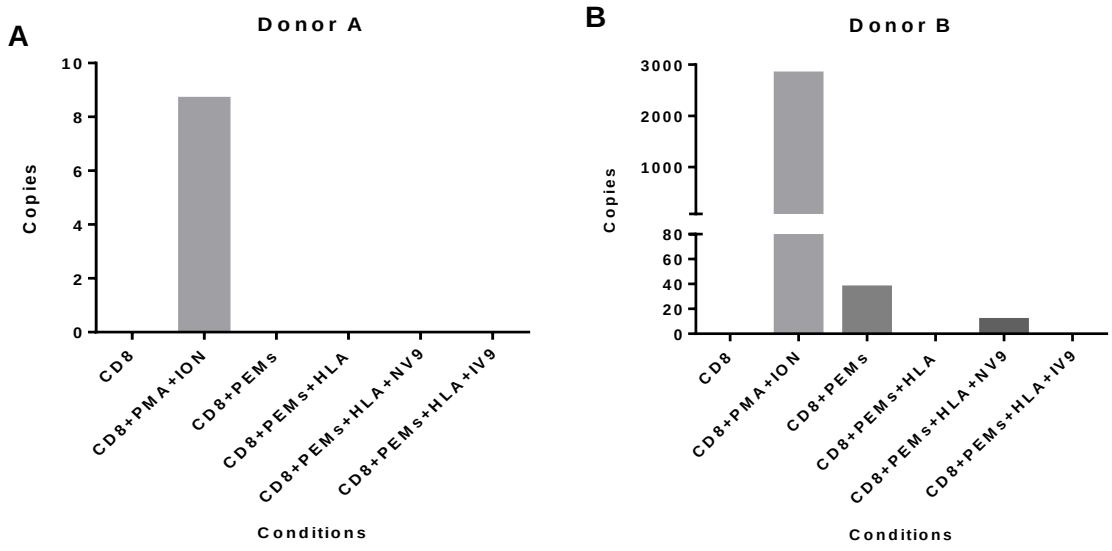
T cells. D – Cells isolated from donor D. 90.5% were CD8⁺ T cells. E – Cells isolated from donor E, being 93.6% of the cells CD8⁺ T cells. F - Cell– isolated from donor F. 87.9% of the cells isolated were CD8⁺ T cells.

The results obtained by flow cytometry to verify the efficacy of isolation method are detailed in Figure 3.2. An aliquot of cells from isolation was marked with two different antibodies that are specific for CD8⁺ cells. In the experiments, the antibodies used were anti-CD3 allophycocyanin (APC) conjugated, which is specific for lymphocytes, and anti-CD8 peridinin-chlorophyll-protein (PerCp), which is specific for CD8⁺ cells. Through analysis of the results obtained, it is possible to verify that the majority of the cells that were isolated are CD8⁺ T cells. Since CD8⁺ T cells are stained using two antibodies, these cells appear in Q2 quadrant of the graphs presented in Figure 3.2. Figure 3.2A shows an efficacy of 94.4%, since 94.4% of the cells that were isolated were CD8⁺ cells. The isolation of CD8⁺ cells from donor B (Figure 3.2B) led to an efficacy of 86.3% and from donor C (Figure 3.2C) of 84.6%. Figure 3.2D proves that from donor D 90.5% the cells isolated were CD8⁺ cells and Figure 3.2E shows that 93.6% of the cells isolated from donor E were CD8⁺ cells. Finally, we can also see that from donor F 87.9% (Figure 3.2F) of the cells that were isolated from the buffy coat, there were the interested cells – CD8⁺ cells.

All these results permit to conclude that the method of the density gradient followed by immunomagnetic separation for isolating CD8⁺ cells from the buffy coats was successful since the purity of CD8⁺ cells isolated for each donor was on average above > 89.7% ± 3,7%.

3.3 Analysis of 24 hours genetic expression of IFN-γ by T cells

Firstly, co-cultures of PEMs with CD8⁺ cells were maintained for 24 hours. After this period, the supernatant and the cells were collected. The cells were lysed and the ribonucleic acid (RNA) was extracted, further converted into complementary deoxyribonucleic acid (cDNA) and the expression of IFN-γ was studied by quantitative polymerase chain reaction (qPCR). Figure 3.3 presents the results that were obtained.



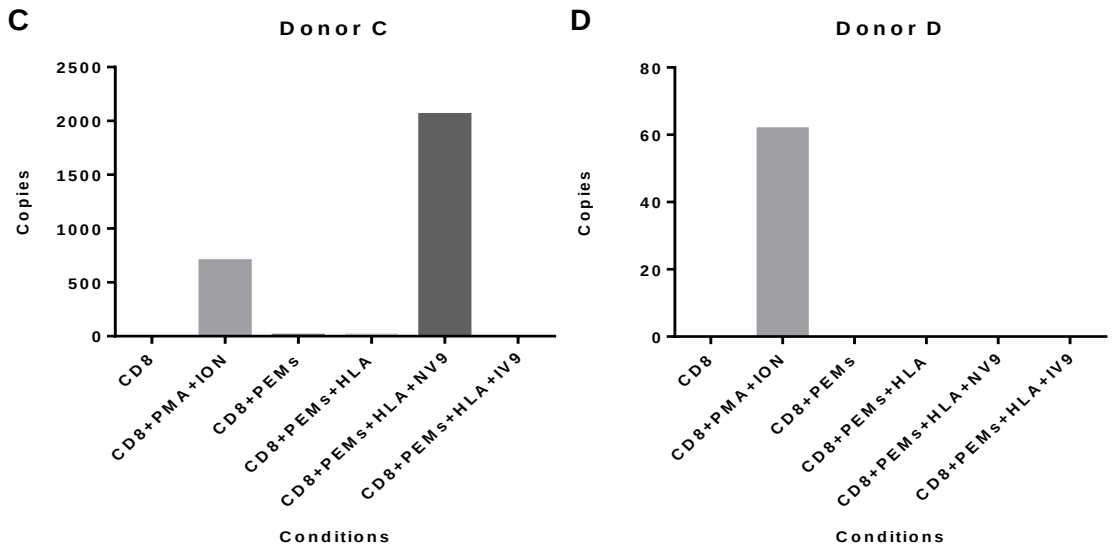


Figure 3.3 - Genetic expression of IFN-γ measured by RT-PCR in the cells recovered from co-cultures experiments at 24 hours. *In the graphs, it is shown the relative mRNA expression of IFN-γ gene. The yy axis represents the relative mRNA levels and the xx axis represents the different conditions studied. CD8 – $CD8^+$ T cells were cultured alone. CD8+PMA+ION – $CD8^+$ T cells were stimulated with PMA and Ionomycin (positive control). CD8+PEMs – $CD8^+$ T cells were co-cultured with PEMs (microcapsules). CD8+PEMs+HLA – $CD8^+$ T cells were put in culture with PEMs linked to HLA-A02 molecules without any peptide. CD8+PEMs+HLA+NV9 – $CD8^+$ T cells were co-cultured with PEMs linked to HLA-A02 molecules complexed with NV9 peptide (CMV peptide). CD8+PEMs+HLA+IV9 – $CD8^+$ T cells were cultured with PEMs linked to HLA-A02 molecules complexed with the IV9 peptide (HIV peptide – irrelevant control). A - Relative mRNA expression of IFN-γ of donor A. B - Relative mRNA expression of IFN-γ of donor B. C - Relative mRNA expression of IFN-γ of donor C. D - Relative mRNA expression of IFN-γ of donor D.*

In all donors (A, B, C and D) it is possible to verify that positive controls worked (Figure 3.3 second column of all graphs), because there was a great increase of IFN-γ expression when the cells were put in culture in the presence of Phorbol 12-myristate 13-acetate (PMA) and Ionomycin (Ion).

In donor A (Figure 3.3A) there was expression of IFN-γ only in positive control. This was expected since donor A was HLA-A02 negative and all the PEMs technology was optimized for HLA-A02 positive donors.

In donor B (Figure 3.3B) there was a very small increase of the expression of IFN-γ when the $CD8^+$ cells were cultured with the presence of the PEMs (third column) and when the cells were co-cultured with PEMs with HLA-A02 complexed with the NV9 peptide (fifth column). Donor C (Figure 3.3C) presented a great increase of the IFN-γ expression when the cells where cultured with PEMs with HLA-A02 complexed with NV9 and donor D (Figure 3.3D) only expressed IFN-γ in positive control.

Since donors B, C and D were HLA-A02 positive, an increase of the expression of IFN-γ when the $CD8^+$ cells were cultured with PEMs with HLA-A02 molecules complexed with the NV9 peptide was expected. This fact only happened with significance in donor C probably because it

was the only CMV positive donor. Since the donors were not tested for CMV, it was not possible to know in advance if the donors were responsive or not to the CMV peptide.

In four donors non-stimulated cells ($CD8^+$ cells alone) didn't express IFN-γ. As positive control, PMA+Ion stimulated cells expressed 899.0 ± 661.9 copies of IFN-γ. Cells stimulated with PEMs alone expressed 10.64 ± 8.9 copies of IFN-γ and cells stimulated with PEMs complexed with HLA-A02 molecules expressed 2.5 ± 2.5 copies. $CD8^+$ cells stimulated with PEMs complexed with NV9-HLA-A02 molecules expressed 516.8 ± 513.1 copies and the cells stimulated with PEMs complexed with IV9-HLA-A02 molecules expressed 0.03 ± 0.03 copies of IFN-γ.

3.4 Analysis of 24 hours expression of IFN-γ protein by T cells

To confirm the results that were obtained with qPCR, an Enzyme-Linked Immunosorbent Assay (ELISA) technique was performed. For ELISA there were used the supernatants collected from the co-culture after 24 hours. The results obtained by ELISA are shown in Figure 3.4.

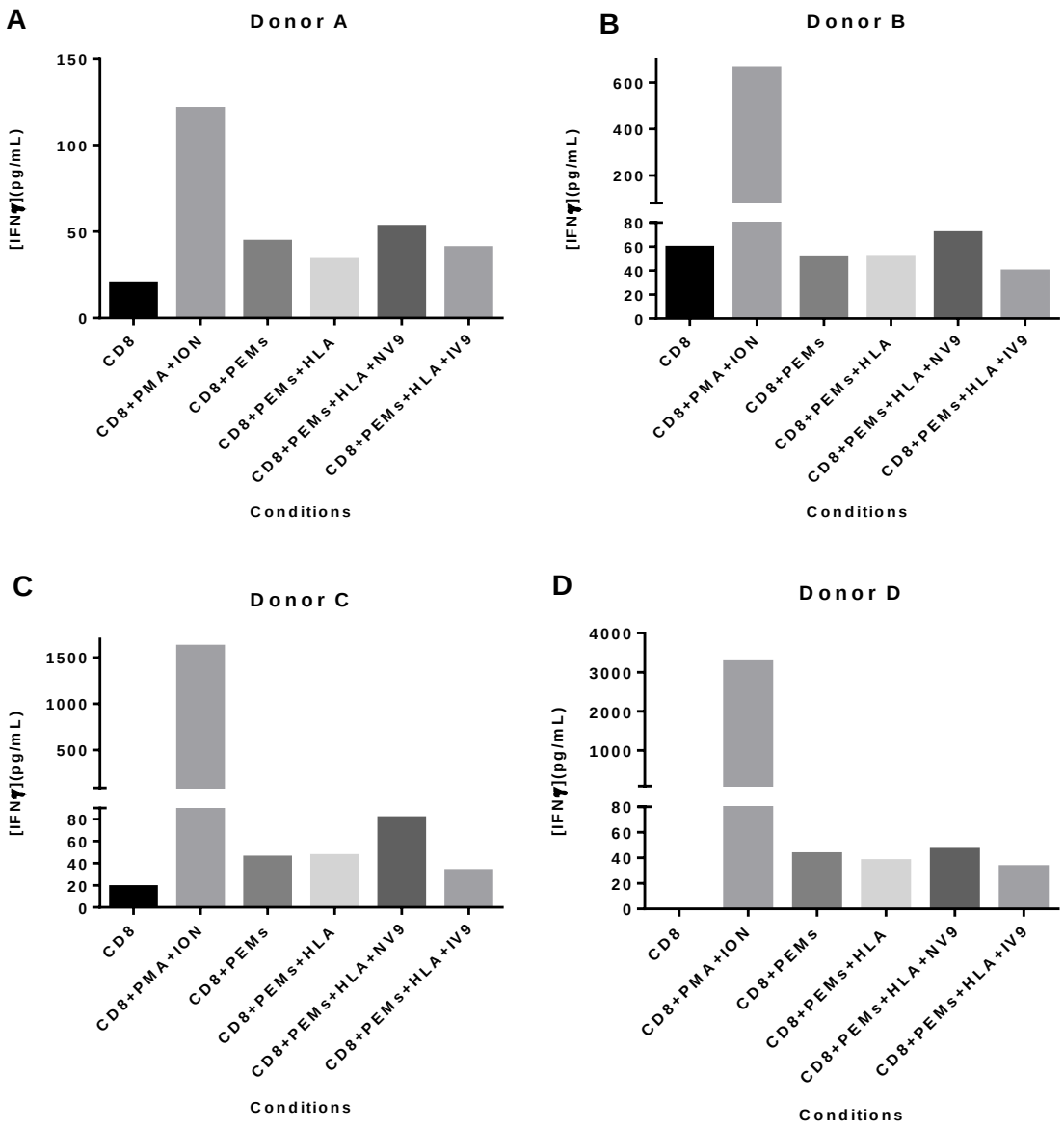


Figure 3.4 – Expression of IFN-γ measured by ELISA in the supernatants recovered from co-cultures experiments at 24 hours. *In the graphs it is shown the concentration (pg/mL) of IFN-γ. The yy axis represents the concentration in pg/mL of IFN-γ in the supernatants and the xx axis represents the different conditions studied. CD8 – $CD8^+$ T cells were cultured alone. CD8+PMA+ION – $CD8^+$ T cells were stimulated with PMA and Ionomycin (positive control). CD8+PEMs – $CD8^+$ T cells were co-cultured with PEMs (microcapsules). CD8+PEMs+HLA – $CD8^+$ T cells were put in culture with PEMs linked to HLA-A02 molecules without any peptide. CD8+PEMs+HLA+NV9 – $CD8^+$ T cells were co-cultured with PEMs linked to HLA-A02 molecules complexed with NV9 peptide (CMV peptide). CD8+PEMs+HLA+IV9 – $CD8^+$ T cells were cultured with PEMs linked to HLA-A02 molecules complexed with the IV9 peptide (HIV peptide – irrelevant control). A – Concentration of IFN-γ on supernatants from donor A. B - Concentration of IFN-γ on supernatants from donor B. C - Concentration of IFN-γ on supernatants from donor C. D - Concentration of IFN-γ on supernatants from donor D.*

The results obtained show an increase of the secretion of IFN-γ in positive controls (second column) in all donors (Figure 3.4A, B, C and D). It is also clear a tendency of positive expression in all other conditions in all donors but it is not possible to see a bigger expression when the cells were cultured with the presence of PEMs with HLA-A02 complexed with NV9 even in donor C where it was expected to be seen.

Non-stimulated cells expressed 24.5 ± 12.5 pg/mL of IFN-γ. Positive controls, cells stimulated with PMA+Ion, expressed 1413 ± 686.6 pg/mL of IFN-γ and cells stimulated with PEMs expressed 45.6 ± 1.7 pg/mL of IFN-γ. Cells that were stimulated with PEMs complexed with HLA-A02 molecules expressed 42.1 ± 3.9 pg/mL of IFN-γ. CD8+ cells stimulated with PEMs complexed with NV9-HLA-A02 molecules expressed 62.8 ± 8.0 pg/mL of IFN-γ and cells stimulated with PEMs complexed with IV9-HLA-A02 molecules expressed 36.4 ± 2.1 pg/mL of IFN-γ.

With these results, it was hypothesized that probably cells need to be stimulated for longer periods of time with PEMs, so that these ones can activate them via signal 1 and the cells produce and release the sufficient amounts of IFN-γ to be detected in supernatants by ELISA. For this reason the co-culture time was increased to 72 hours.

3.5 Analysis of 72 hours genetic expression of IFN-γ by T cells

As mentioned before the time of co-cultures was increased to 72 hours being all the procedures maintained. So, after 72 hours of co-culture, the supernatant was collected and the cells lysed and the RNA extracted. After conversion of the RNA for cDNA the genetic expression was analyzed by qPCR. The results obtained are in Figure 3.5.

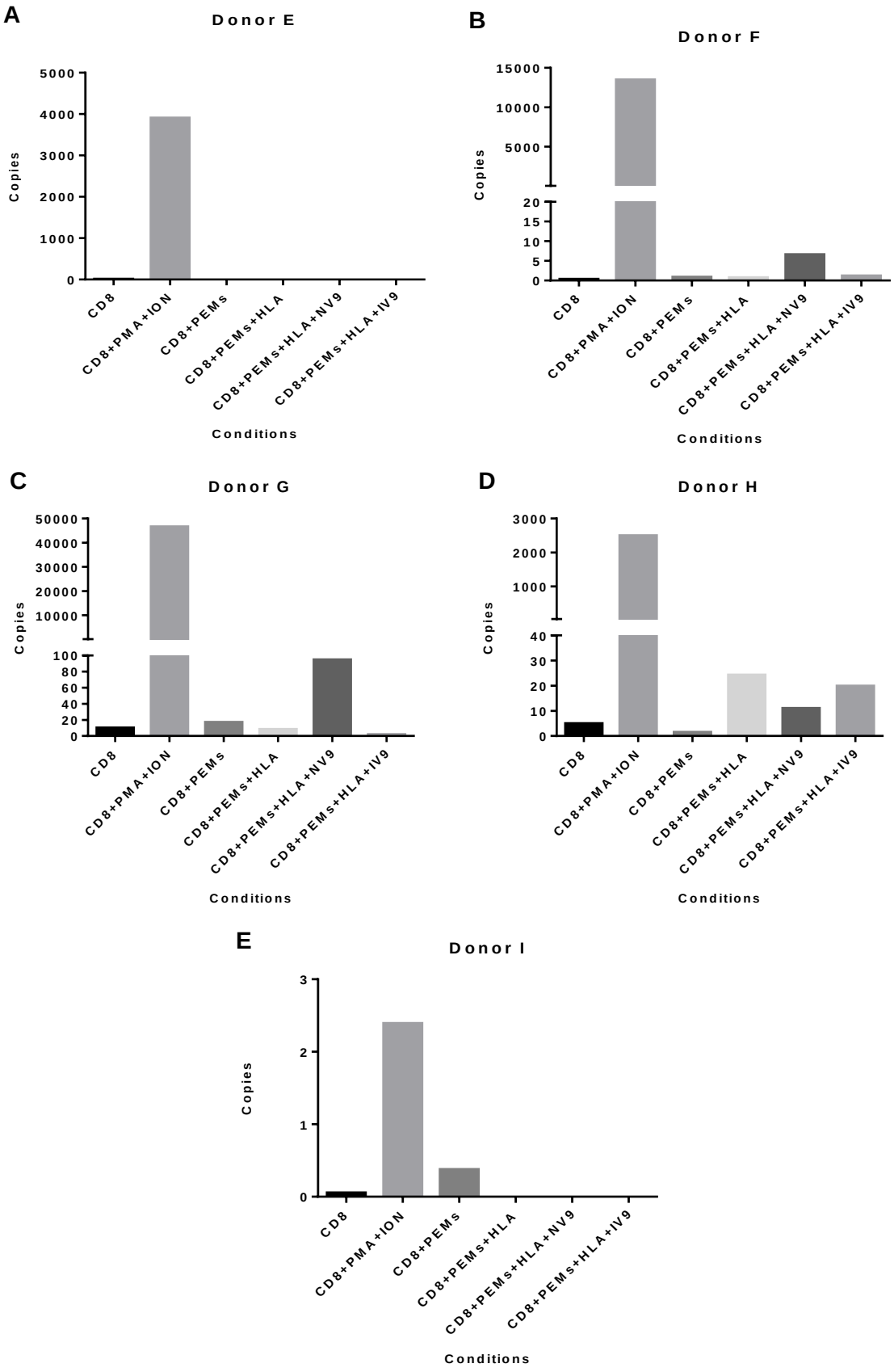


Figure 3.5 - Genetic expression of IFN-γ measured by RT-PCR in the cells recovered from co-cultures experiments at 72 hours. *In the graphs it is shown the relative mRNA expression of IFN-γ gene.*

The yy axis represents the relative mRNA levels and the xx axis represents the different conditions studied. CD8 – $CD8^+$ T cells were cultured alone. CD8+PMA+ION – $CD8^+$ T cells were stimulated with PMA and Ionomycin (positive control). CD8+PEMs – $CD8^+$ T cells were co-cultured with PEMs (microcapsules). CD8+PEMs+HLA – $CD8^+$ T cells were put in culture with PEMs linked to HLA-A02 molecules without any peptide. CD8+PEMs+HLA+NV9 – $CD8^+$ T cells were co-cultured with PEMs linked to HLA-A02 molecules complexed with NV9 peptide (CMV peptide). CD8+PEMs+HLA+IV9 – $CD8^+$ T cells were cultured with PEMs linked to HLA-A02 molecules complexed with the IV9 peptide (HIV peptide – irrelevant control). A - Relative mRNA expression of IFN-γ of donor E. B - Relative mRNA expression of IFN-γ of donor F. C - Relative mRNA expression of IFN-γ of donor G. D - Relative mRNA expression of IFN-γ of donor H. E - Relative mRNA expression of IFN-γ of donor I.

Analyzing the results obtained by qPCR, it is possible to confirm that the positive controls (second column of all graphs – Figure 3.5) are reproducible, since there was an increase of the expression of IFN-γ in all donors (E, F, G, H and I) when the cells were stimulated with PMA and Ion.

In donor E (Figure 3.5A) there wasn't any expression in other conditions than positive control. This could be due to the fact that the donor wasn't CMV positive like it was mentioned before.

Donor F (Figure 3.5B) and donor H (Figure 3.5D) present a small increase (less than 20 copies) of the expression of IFN-γ in the other conditions that are not significant when compared to the expression in the positive control.

Donor G (Figure 3.5C), besides positive control, shows increased expression of IFN-γ when the cells where co-cultured with PEMs with HLA-A02 molecules complexed with CMV peptide (fifth column). This result could indicate that donor G was CMV-responsive and T cells were activated by PEMs technology.

Donor I (Figure 3.5E), presents a small increase of the IFN-γ expression when cells were co-cultured with PEMs alone (third column).

All together these results show that non-stimulated cells expressed 4.2 ± 1.8 copies of IFN-γ. $CD8^+$ cells stimulated with PMA+Ion expressed 13275 ± 8620 copies of IFN-γ and the ones stimulated with PEMs alone expressed 4.4 ± 3.1 copies. Cells that were stimulated with PEMs complexed with HLA-A02 molecules expressed 7.2 ± 4.5 copies of IFN-γ and cells stimulated with PEMs complexed with NV9-HLA-A02 molecules expressed 22.4 ± 18.2 copies. $CD8^+$ cells stimulated with PEMs complexed with IV9-HLA-A02 molecules expressed 5.2 ± 3.7 copies of IFN-γ.

Increase of the expression of IFN-γ in other conditions when the CMV peptide is not present, can happen because PEMs in culture with the cells can interfere with some activation mechanisms (basal activation) and a small expression is noticed.

3.6 Analysis of 72 hours expression of IFN-γ protein by T cells

The qPCR results from previous section were compared to the results obtained using the ELISA technique. ELISA results are presented in Figure 3.6.

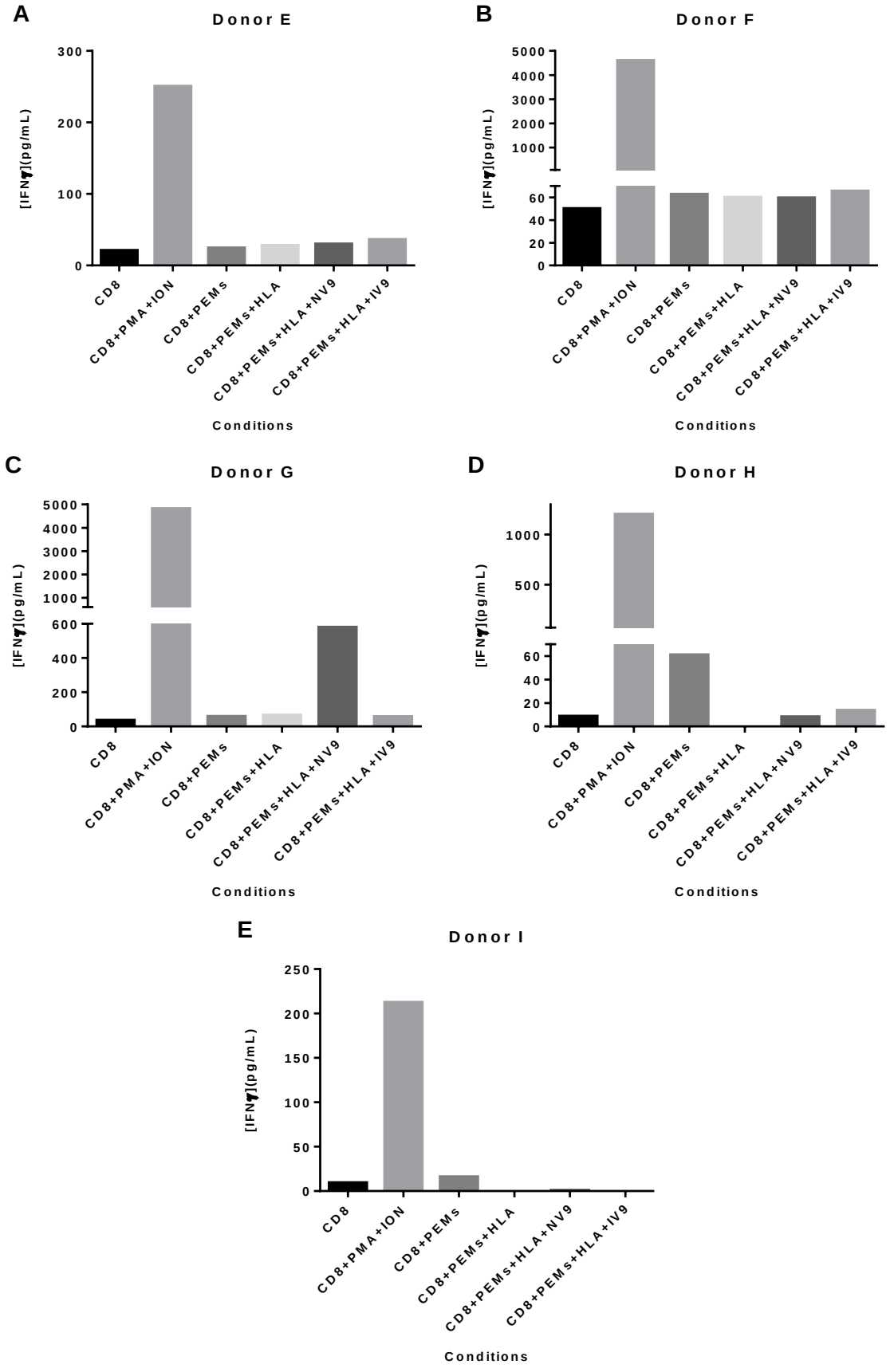


Figure 3.6 - Expression of IFN-γ measured by ELISA in the supernatants recovered from co-cultures experiments at 72 hours. *In the graphs it is shown the concentration (pg/mL) of IFN-γ. The yy axis represents the concentration in pg/mL of IFN-γ in the supernatants and the xx axis represents the different conditions studied. CD8 – $CD8^+$ T cells were cultured alone. CD8+PMA+ION – $CD8^+$ T cells were stimulated with PMA and Ionomycin (positive control). CD8+PEMs – $CD8^+$ T cells were co-cultured with PEMs (microcapsules). CD8+PEMs+HLA – $CD8^+$ T cells were put in culture with PEMs linked to HLA-A02 molecules without any peptide. CD8+PEMs+HLA+NV9 – $CD8^+$ T cells were co-cultured with PEMs linked to HLA-A02 molecules complexed with NV9 peptide (CMV peptide). CD8+PEMs+HLA+IV9 – $CD8^+$ T cells were cultured with PEMs linked to HLA-A02 molecules complexed with the IV9 peptide (HIV peptide – irrelevant control). A – Concentration of IFN-γ on supernatants from donor E. B - Concentration of IFN-γ on supernatants from donor F. C - Concentration of IFN-γ on supernatants from donor G. D - Concentration of IFN-γ on supernatants from donor H. E - Concentration of IFN-γ on supernatants from donor I.*

With these results, it is possible to confirm that positive controls were working, since there was increased secretion of IFN-γ when cells were stimulated with PMA and Ion. In donors E and F (Figure 3.6A and Figure 3.6B) it was possible to detect reduced levels of IFN-γ produced for all other conditions. It may happen because of the sensitivity of the technique.

In donor G (Figure 3.6C) the results obtained confirm the ones obtained by qPCR, since there was a significant expression when the cells were cultured with PEMs with HLA-A02 complexed with NV9 peptide. Both results (qPCR and ELISA) suggest that donor G was CMV positive and PEMs could activate T cells against the peptide that was presented.

Donor H shows an isolated higher expression when cells were in contact with PEMs alone (Figure 3.6D). Once again, this may indicate that PEMs (since they are strange particles to the cells) can interfere with some signaling pathways and activation mechanisms.

In donor I (Figure 3.6E) the results are the same that the ones obtained by qPCR, showing a small increase when cells were alone in culture and when they were cultured with PEMs alone.

Considering the results of the five donors, non-stimulated cells expressed 24.8 ± 7.9 pg/mL of IFN-γ and cells stimulated with PMA+Ion, expressed 2215 ± 1033 pg/mL of IFN-γ. $CD8^+$ cells stimulated with PEMs expressed 44.3 ± 9.9 pg/mL of IFN-γ and cells that were stimulated with PEMs complexed with HLA-A02 molecules expressed 30.6 ± 14 pg/mL of IFN-γ. $CD8^+$ cells stimulated with PEMs complexed with NV9-HLA-A02 molecules expressed 135.5 ± 111.4 pg/mL of IFN-γ and cells stimulated with PEMs complexed with IV9-HLA-A02 molecules expressed 34.4 ± 12.4 pg/mL of IFN-γ.

All the results obtained by qPCR and ELISA from all co-cultures suggest that PEMs with HLA-A02 molecules complexed with CMV peptide can activate T cells from donors, supposing they had previous contact with the CMV virus and therefore have CMV-reactive lymphocytes. It also indicates that PEMs are microcapsules that are not very stable and can interfere with some signaling mechanisms of the cells inducing unspecific increases of the expression of IFN-γ.

3.7 Confirmation of the binding between PEMs and CD8 cells

To confirm that PEMs can bind to $CD8^+$ cells, we set up a method that allowed the identification of PEMs by flow cytometry. The methodology used to perform these tests is described in section 2.10 and to analyze the results obtained, it was used the gate strategy illustrated in Figure 3.7.

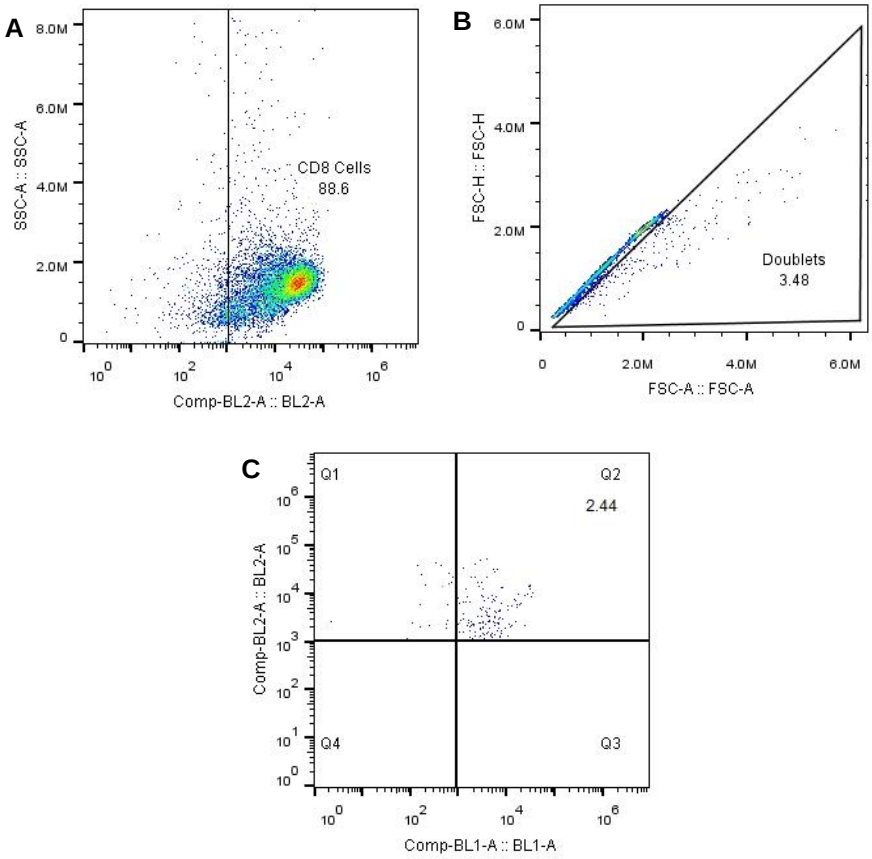


Figure 3.7 - Gate strategy used to test the binding between PEMs and CD8 T cells. *A – First, there were selected only the events stained with anti-CD8 PE. B – From anti-CD8 PE stained events, it was selected only the doublets. For this purpose, we performed a FSC-A:FSC-H dot plot and selected the events that were not in the diagonal. C-Finally the two fluorescences were intersected and the doublets double stained with biotin FITC and anti-CD8 PE correspond to PEMs binding with $CD8^+$ cells.*

Firstly, the gating strategy selected the eventsstained with anti-CD8 phycoerythrin (PE) – Figure 3.7A. From the anti-CD8 PE stained events, only the doublets that could be PEMs-PEMs, PEMs-$CD8^+$ cells or $CD8^+$ cell-$CD8^+$ cells were selected (Figure 3.7B).. The gating selection included the doublets that were double stained, that is, stained with both biotin FITC and anti-CD8 PE (Figure 3.7C). These doublets double stained correspond to the PEMs binding to CD8+ cells.

Controls were first analyzed and the results are shown in Figure 3.8.

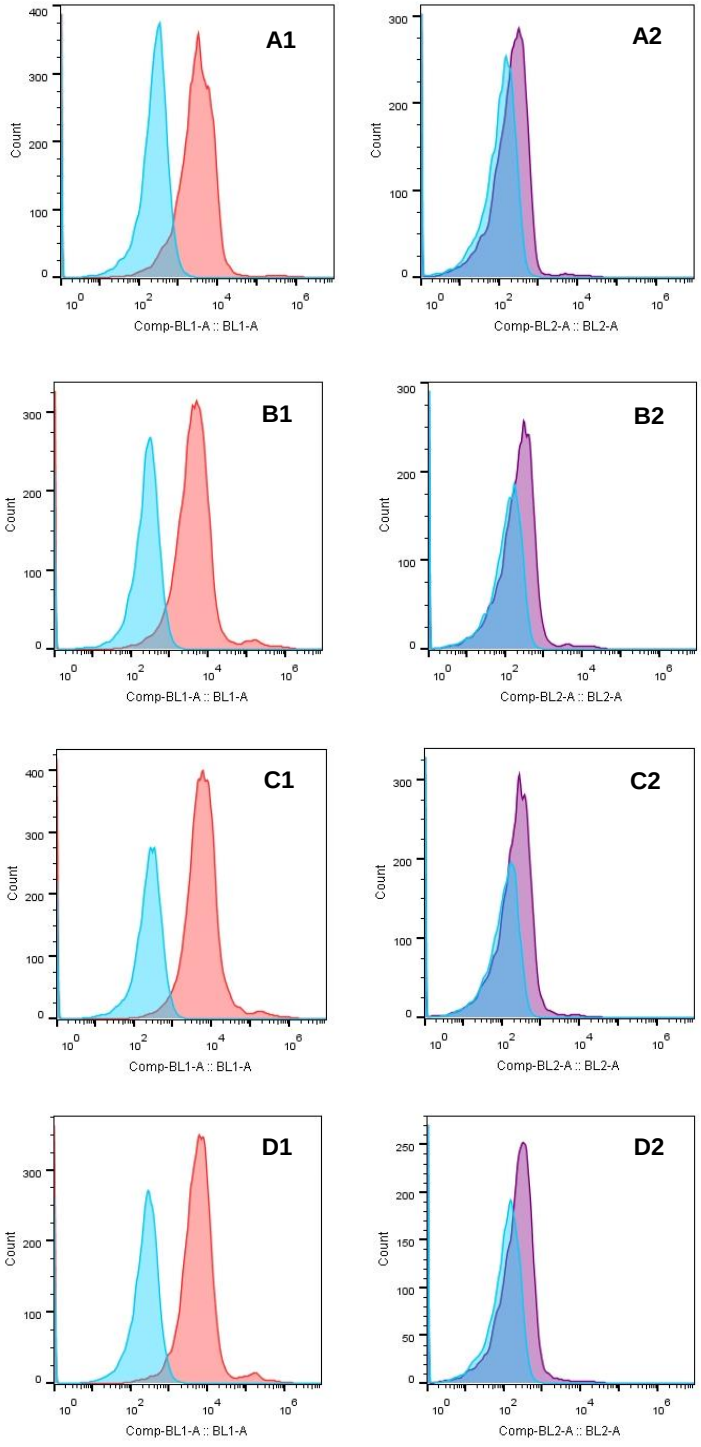


Figure 3.8 - PEMs in the different conditions stained with biotin FITC and CD8 PE. *A1 – PEMs stained with biotin FITC (red) and unstained cells (blue). A2 – PEMs stained with CD8 PE (purple) and unstained cells (blue). B1 – PEMs with HLA-A02 stained with biotin FITC (red) and unstained cells (blue). B2 - PEMs with HLA-A02 stained with CD8 PE (purple) and unstained cells (blue). C1 – PEMs with HLA-A02 complexed with NV9 peptide stained with biotin FITC (red) and unstained cells (blue). C2 - PEMs with HLA-A02 complexed with NV9 peptide stained with CD8 PE (purple) and unstained cells (blue). D1 – PEMs*

with HLA-A02 complexed with IV9 peptide stained with biotin FITC (red) and unstained cells (blue). D2 - PEMs with HLA-A02 complexed with IV9 peptide stained with CD8 PE (purple) and unstained cells (blue).

Figure 3.8A corresponds to PEMs stained with biotin-FITC and anti-CD8 PE. In Figure 3.8B it is shown the results obtained for PEMs with HLA-A02 molecules stained with biotin-FITC and anti-CD8 PE. Results from PEMs with HLA-A02 molecules complexed with NV9 peptide and PEMs with HLA-A02 molecules complexed with IV9 peptide both stained with biotin FITC and anti-CD8 PE are represented in Figures 3.8C and 3.8D respectively.

These results show that in all the situations tested, PEMs are stained with biotin-FITC (Figures 3.8A1, 3.8B1, 3.8C1 and 3.8D1) but not with anti-CD8 PE (Figures 3.8A2, 3.8B2, 3.8C2 and 3.8D2). All the results obtained were expected, since PEMs are coated with streptavidin, and this molecule binds naturally to biotin molecules. Otherwise, PEMs are microcapsules that do not have CD8 molecules, that's why the antibody CD8 PE didn't bind to the PEMs in any condition. With these controls it was possible to guarantee that the biotin FITC binds to PEMs in any condition and the antibody CD8 PE didn't bind to PEMs in any condition.

After this, it was possible to analyze the binding between PEMs and $CD8^+$ cells after 24 hours of co-culture. To do these experiments there were used $CD8^+$ cells from three different donors and the results are in Figures 3.9, 3.10 and 3.11.

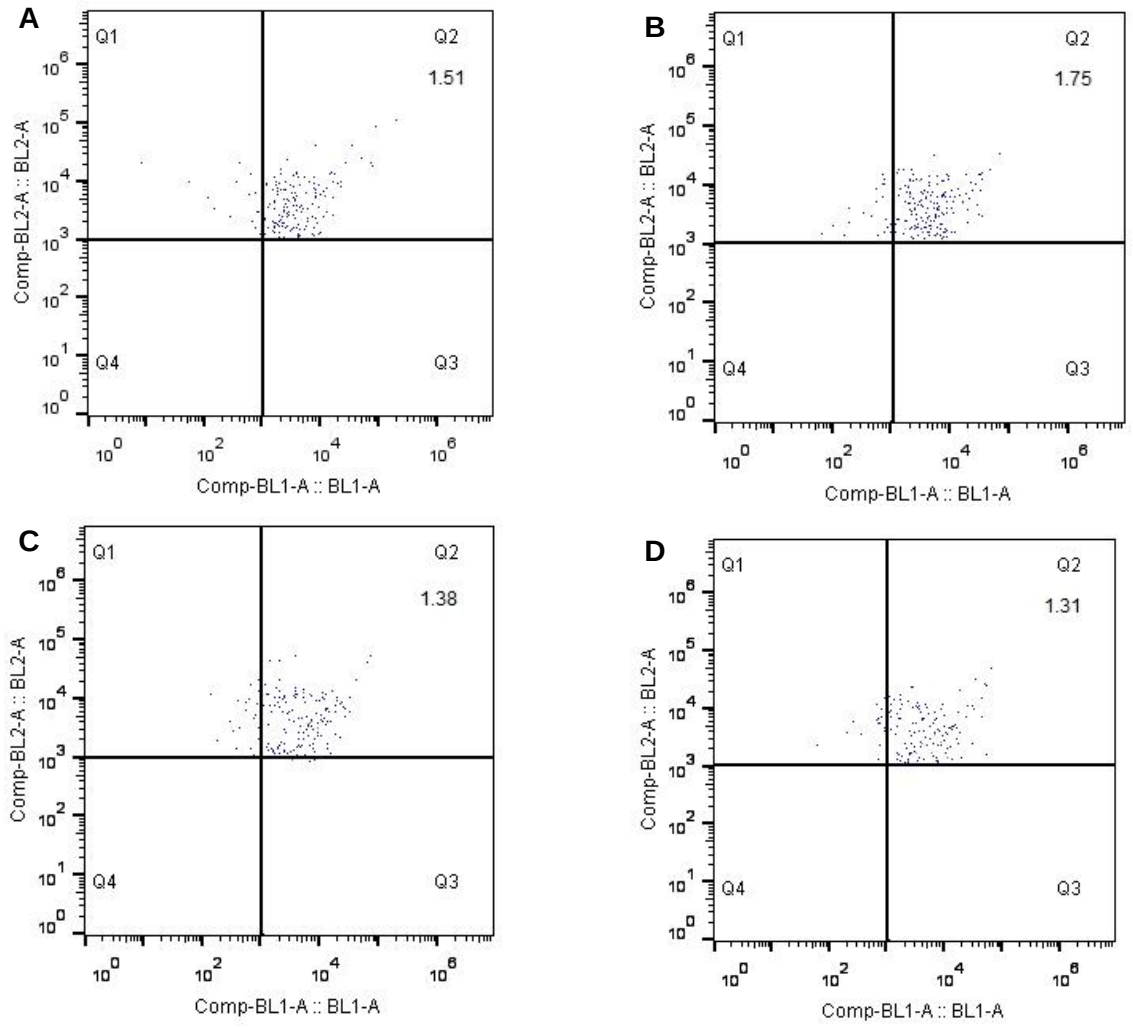
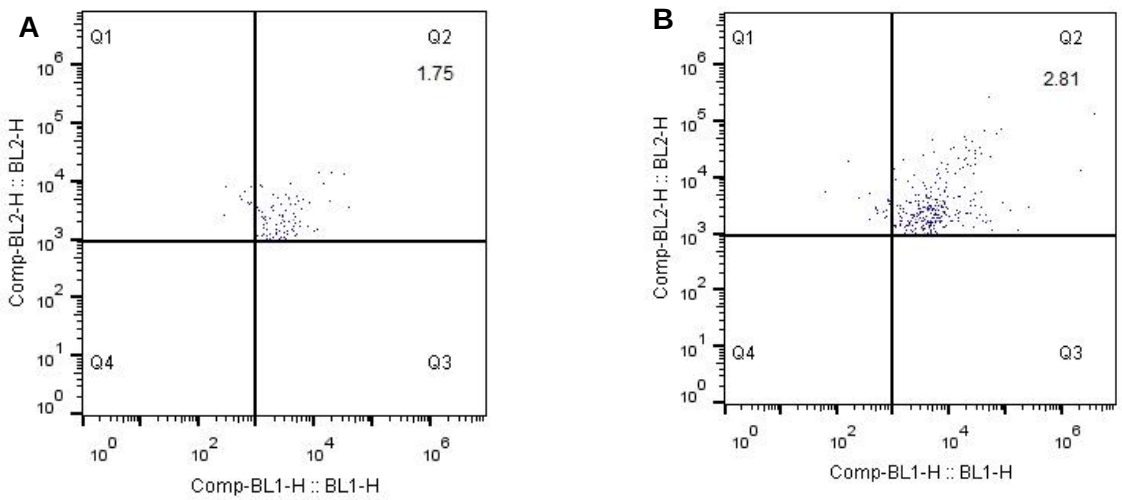


Figure 3.9 – Binding between PEMs and CD8 cells analyzed by flow cytometry. *In Q2 quadrant are the doublets (PEMs+CD8) double stained with biotin FITC and CD8 PE. $CD8^+$ T cells used in co-culture were from donor G. A – Results from co-culture of PEMs (just the microcapsules) and $CD8^+$ cells. B – Results from co-culture of PEMs with HLA-A02 with $CD8^+$ cells. C- Results from co-culture of PEMs with HLA-A02 complexed with NV9 peptide with $CD8^+$ cells. D - Results from co-culture of PEMs with HLA-A02 complexed with IV9 peptide with $CD8^+$ cells.*

Figure 3.9 shows the results obtained by flow cytometry to confirm that PEMs can bind to $CD8^+$ cells. $CD8^+$ cells used in this assay were from a suppose CMV positive donor. The percentage of binding when $CD8^+$ cells were co-cultured with PEMs alone was 1.51% (Figure 3.9A) and when $CD8^+$ cells were co-cultured with PEMs with HLA-A02 molecules was 1.75% (Figure 3.9B). Figure 3.9C shows that the percentage of binding was 1.38% when $CD8^+$ cells were cultured with PEMs with HLA-A02 molecules complexed with NV9 peptide and Figure 3.9D shows the percentage of binding when $CD8^+$ cells were co-cultured with PEMs with HLA-A02 molecules complexed with IV9 peptide was about 1.31%.

These results were not expected since it was believed that PEMs should bind preferentially when there were HLA-A02 molecules complexed with NV9 peptide in CMV positive donors. Analyzing the results it is possible to see a bigger percentage of binding when the cells were co-cultured with PEMs with HLA-A02 molecules without any peptide (Figure 3.9B). With these results, we can conclude that PEMs bind to $CD8^+$ cells irrespectively of the peptide being presented, showing no differences in percentage of binding from one condition to another. This results suggests that PEMs are not specific since they don't bind to a higher number of $CD8^+$ cells when the NV9 peptide is present.



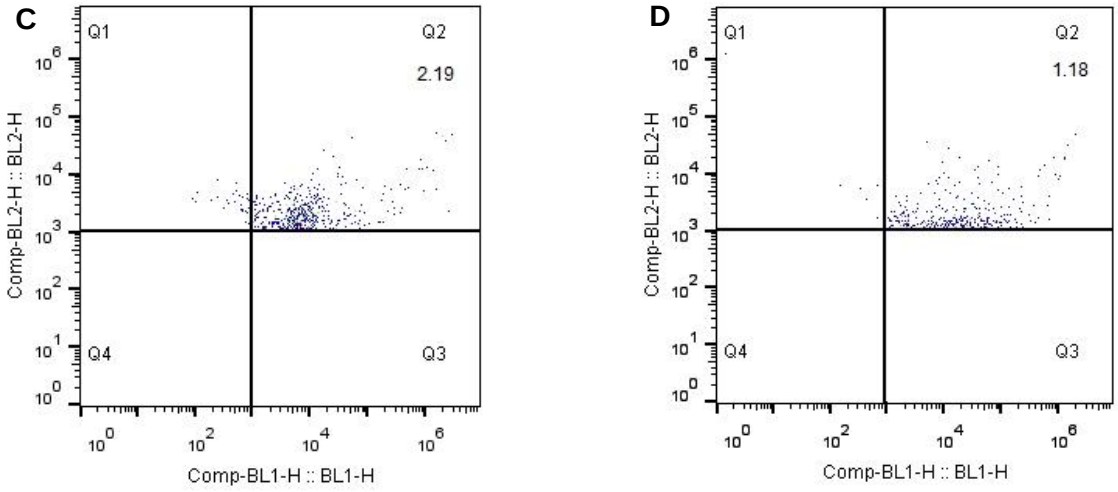


Figure 3.10 - Binding between PEMs and CD8 cells analyzed by flow cytometry. *In Q2 quadrant are the doublets (PEMs+CD8) double stained with biotin FITC and CD8 PE. CD8⁺ T cells used in co-culture were from donor I. A – Results from co-culture of PEMs (just the microcapsules) and CD8⁺ cells. B – Results from co-culture of PEMs with HLA-A02 with CD8⁺ cells. C- Results from co-culture of PEMs with HLA-A02 complexed with NV9 peptide with CD8⁺ cells. D - Results from co-culture of PEMs with HLA-A02 complexed with IV9 peptide with CD8⁺ cells.*

As mentioned before, the assay to confirm that PEMs bind to CD8⁺ T cells was conducted with CD8⁺ cells from three different donors. Figure 3.10 shows the results obtained using CD8⁺ cells from donor I, which was a non-responsive CMV donor. Figure 3.10A shows that the percentage of binding when CD8⁺ cells were co-cultured with PEMs alone was 1.75% and when CD8⁺ cells were co-cultured with PEMs with HLA-A02 molecules was 2.81% (Figure 3.10B). Figure 3.10C shows that the percentage of binding was 2.19% when CD8⁺ cells were cultured with PEMs with HLA-A02 molecules complexed with NV9 peptide and Figure 3.10D shows the percentage of binding when CD8⁺ cells were co-cultured with PEMs with HLA-02 molecules complexed with IV9 peptide was about 1.18%.

Similarly, as with the previous section, these results were not expected since it is possible to observe a higher percentage of binding when CD8⁺ cells were co-cultured with PEMs with HLA-A02 molecules (Figure 3.10B) and besides CD8⁺ cells were from a non CMV donor it was possible to confirm thatCD8⁺ cells and PEMs form a bond when cells were co-cultured with PEMs with HLA-A02 molecules complexed with NV9 peptide (Figure 3.10C) which was not expected. Comparing the results from this donor with the results from a CMV positive donor (Figure 3.9), it is not possible to see considerable differences (proportionally), which further suggest that PEMs are not able to drive antigen-specific responses.

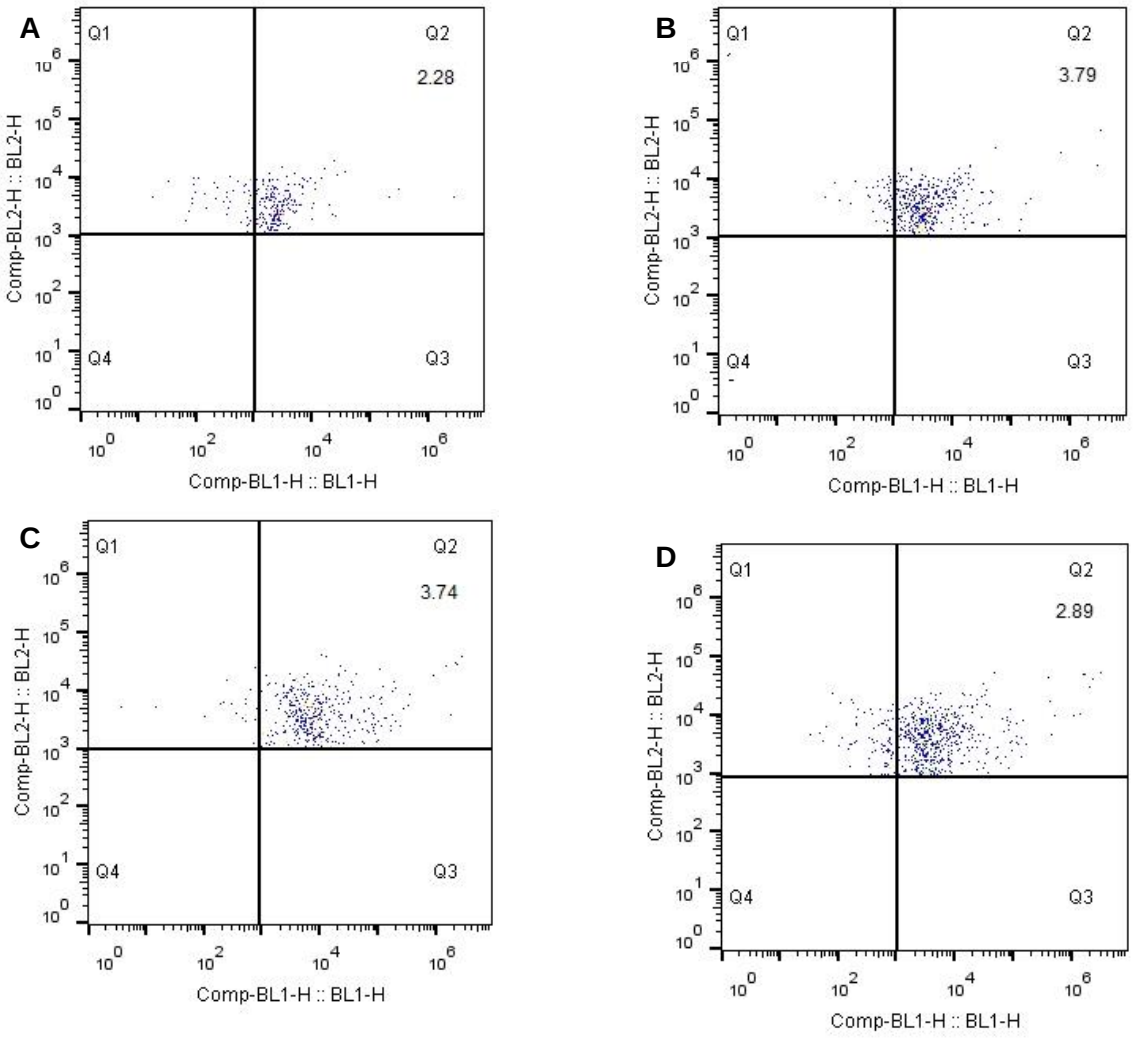


Figure 3.11 - Binding between PEMs and CD8 cells analyzed by flow cytometry. *In Q2 quadrant are the doublets (PEMs+CD8) double stained with biotin FITC and CD8 PE. $CD8^+$ T cells used in co-culture were from donor H. A – Results from co-culture of PEMs (just the microcapsules) and $CD8^+$ cells. B – Results from co-culture of PEMs with HLA-A02 with $CD8^+$ cells. C- Results from co-culture of PEMs with HLA-A02 complexed with NV9 peptide with $CD8^+$ cells. D - Results from co-culture of PEMs with HLA-A02 complexed with IV9 peptide with $CD8^+$ cells.*

The results obtained using the $CD8^+$ cells from the third donor (donor H) are shown in Figure 3.11. H donor was not responsive to CMV peptide as it was observed in qPCR and ELISA results. The percentage of binding when $CD8^+$ cells were co-cultured with PEMs alone was 2.28% (Figure 3.11A). Figure 3.11B shows that the percentage of binding when $CD8^+$ cells were co-cultured with PEMs with HLA-A02 molecules was 3.79% and Figure 3.11C shows that the percentage of binding was 3.74% when $CD8^+$ cells were cultured with PEMs with HLA-A02 molecules complexed with NV9 peptide. Finally, the percentage of binding when $CD8^+$ cells were co-cultured with PEMs with HLA-02 molecules complexed with IV9 peptide was about 2.89% (Figure 3.11D).

As the results shown before, these results were not expected since it is possible to observe, once again, a bigger percentage of binding when $CD8^+$ cells were co-cultured with PEMs with

HLA-A02 molecules (Figure 3.11B) and a link between the cells and microcapsules when cells were co-cultured with PEMs with HLA-A02 molecules complexed with NV9 peptide (Figure 3.11C) besides cells being from a non CMV donor.

Considering the results from the three donors, it is also seen (in all donors) that $CD8^+$ cells bind to PEMs with HLA-A02 molecules complexed with IV9 peptide (Figures 3.9D, 3.10D and 3.11D). Considering that only a small percentage (less than 5%) of cells were interacting with PEMs, this was not expected because $CD8^+$ cells were isolated from a healthy donor that should not respond to the HIV peptide. All these results together confirm that PEMs are not stable and are not specific, explaining why they can bind to cells from CMV negative donors and even without having a peptide complexed to them.

4 Conclusion and Future Perspectives

Cancer is one of the most prevalent diseases of the 21st century and one of the leading causes of death around the world. For this reason, it is imperative to try to understand more about the disease and especially about the treatments that can fight it. For this purpose, immunotherapies such as cancer vaccines are one of the approaches that have been studied in the last few decades.

The main aim of this thesis was to test the capacity of PEMs to activate $CD8^+$ T cells, as a possible technology for future therapeutic applications, either as a vaccine or as adjuvant.

Considering all the results, it is possible to confirm that PEMs can activate T cells when complexed with a peptide that T cells where already exposed to.

In contrast, it is also true that PEMs are unstable microcapsules that can interfere with some mechanisms of the cells.

Since PEMs can only provide the first signal to activate T cells it is relevant to test if the microcapsules provide the other two signals. Performing cell co-cultures with IL-2 in the medium is a possibility in order to provide the third signal. The second signal could also be provided by using antibodies against the co-stimulatory receptors.

Another improvement to the protocol described here should include the screening of CMV-positive donors before co-cultures. Like this, it is possible to confirm that the increase of the expression of IFN-γ was due to the activation of the cells and not due to another factors.

Since our collaborators from Germany confirmed the variability of PEMs due to their manufacture process in other assays, the microcapsules currently being manufactured show increased stability because they present a silica core. These new capsules should also be tested using the same procedures and the results compared to the previous ones to confirm which ones are more stable and better to perform the next tests.

The main goal of these microcapsules is to be used as anticancer immunotherapy. To test them we need to test the capacity of PEMs bind to cancer antigens and activate T cells against these antigens. All these tests should be done in the future.

5 References

[1] A. K. Abbas, A. H. Lichtman, and S. Pillai, “Properties and Overview of Immune Responses,” in *Cellular and Molecular Immunology*, 9th ed., Elsevier, 2017, pp. 1–11.

[2] J. A. Owen, J. Punt, S. A. Stranford, and P. P. Jones, “Overview of the Immune System,” in *Kuby Immunology*, 7th ed., W.H. Freeman & Company, 2013, pp. 1–26.

[3] R. A. Goldsby, T. J. Kindt, J. Kuby, and B. A. Osborne, “Overview of the Immune System,” in *Immunology*, 5th ed., W.H. Freeman, 2002, pp. 1–23.

[4] R. Clark and T. Kupper, “Old meets new: The interaction between innate and adaptive immunity,” *J. Invest. Dermatol.*, vol. 125, no. 4, pp. 629–637, 2005.

[5] A. Abbas and A. Lichtman, “Imunidade Inata,” in *Imunologia Celular e Molecular*, 2nd ed., Saunders, 2008, pp. 23–43.

[6] P. M. Lydyard, A. Whelan, and M. W. Fanger, *Instant Notes:Immunology*, 2nd ed., vol. 76, no. 898. BIOS Scientific, 2004.

[7] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter, “Innate Imunity,” in *Molecular Biology of the Cell*, New York: Garland Science, 2002.

[8] S. Akira, S. Uematsu, and O. Takeuchi, “Pathogen recognition and innate immunity,” *Cell*, vol. 124, no. 4, pp. 783–801, 2006.

[9] C. Alexander and E. T. Rietschel, “Bacterial lipopolysaccharides and innate immunity.,” *J. Endotoxin Res.*, vol. 7, no. 3, pp. 167–202, 2001.

[10] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter, “The Adaptive Immune System,” in *Molecular Biology of the Cell*, 4th ed., Garland Science, 2008, pp. 1539–1601.

[11] C. A. J. Janeway, P. Travers, M. Walport, and M. Shlomchik, “Principles of innate and adaptive immunity,” in *Immunobiology: The Immune System in Health and Disease.*, 5th ed., Garland Science, 2001, pp. 1–9.

[12] F. A. Bonilla and H. C. Oettgen, “Adaptive immunity.”

[13] C. A. J. Janeway, P. Travers, M. Walport, and M. Shlomchik, “The Humoral Immune Response,” in *Immunobiology: The Immune System in Health and Disease.*, Garland Science, 2001.

[14] J. Charles A Janeway, P. Travers, M. Walport, and M. J. Shlomchik, “T Cell-Mediated Immunity,” in *Immunobiology: The Immune System in Health and Disease.*, Garland Science, 2001.

[15] J. A. Owen, J. Punt, S. A. Stranford, and P. P. Jones, “T-Cell Development,” in *Kuby Immunology*, 7th ed., W.H. Freeman & Company, 2013, pp. 299–327.

[16] J. Charles A Janeway, P. Travers, M. Walport, and M. J. Shlomchik, “Generation of lymphocytes in bone marrow and thymus,” in *Immunobiology: The Immune System in Health and Disease.*, Garland Science, 2001.

[17] M. M. Davis and P. J. Bjorkman, “T-cell antigen receptor genes and T-cell recognition,” *Nature*, vol. 334, no. 6181, pp. 395–402, Aug. 1988.

[18] P. J. R. Ebert, Q.-J. Li, J. B. Huppa, and M. M. Davis, “Functional development of the T cell receptor for antigen.,” *Prog. Mol. Biol. Transl. Sci.*, vol. 92, pp. 65–100, 2010.

[19] L. Chen and D. B. Flies, “Molecular mechanisms of T cell co-stimulation and co-inhibtion,” *Nat. Rev. Immunol.*, vol. 13, no. 4, pp. 227–242, 2013.

[20] S. Goral, “The Three-Signal Hypothesis of Lymphocyte Activation/Targets for Immunosuppression,” *Dial. Transplant.*, vol. 40, no. 1, pp. 14–16, 2011.

[21] N. D. Pennock, J. T. White, E. W. Cross, E. E. Cheney, B. A. Tamburini, and R. M. Kedl, “T cell responses: naive to memory and everything in between,” *AJP Adv. Physiol. Educ.*, vol. 37, no. 4, pp. 273–283, 2013.

[22] J. E. Smith-Garvin, G. A. Koretzky, and M. S. Jordan, “T Cell Activation,” *Annu. Rev. Immunol.*, vol. 27, pp. 591–619, 2009.

[23] A. H. Sharpe and A. K. Abbas, “T-Cell Costimulation - Biology, Therapeutic Potential, and Challenges,” *N. Engl. J. Med.*, vol. 355.10, no. 1, pp. 973–975, 2006.

[24] M. Cavanagh, “T-cell activation | British Society for Immunology.” [Online]. Available: https://www.immunology.org/public-information/bitesized-immunology/systems-and-processes/t-cell-activation. [Accessed: 03-May-2018].

[25] P. T. H. Coates, B. L. Colvin, H. Hackstein, and A. W. Thomson, “The interaction between dendritic cells (DCs) and T cells involves three signals,” *Expert Rev. Mol. Med.*, 2002.

[26] M. A. Santana and F. Esquivel-Guadarrama, “Cell Biology of T Cell Activation and Differentiation,” 2006, pp. 217–274.

[27] J. M. Damsker, A. M. Hansen, and R. R. Caspi, “Th1 and Th17 cells: adversaries and

collaborators.," *Ann. N. Y. Acad. Sci.*, vol. 1183, pp. 211–21, Jan. 2010.
[28] J. M. Cruse *et al.*, "Antigen Presentation," *Immunol. Guideb.*, pp. 267–276, 2004.
[29] D. L. Hamilos, "Antigen Presenting Cells.," *Cell. Immunol.*, vol. 8, no. 2, pp. 98–117, 1989.
[30] M. Cella, F. Sallusto, and A. Lanzavecchia, "Origin, maturation and antigen presenting function of dendritic cells," *Curr. Opin. Immunol.*, vol. 9, no. 1, pp. 10–16, Feb. 1997.
[31] S. C. Knight and A. J. Stagg, "Antigen-presenting cell types," *Cuurent Opin. Immunol.*, vol. 5, pp. 374–382, 1993.
[32] P. Cresswell, "Antigen processing and presentation," *Immunol. Rev.*, vol. 207, pp. 5–7, 2005.
[33] M. Das, S. V Kaveri, and J. Bayry, "Cross-presentation of antigens by dendritic cells: role of autophagy.," *Oncotarget*, vol. 6, no. 30, pp. 28527–8, Oct. 2015.
[34] J. F. Fonteneau *et al.*, "Characterization of the MHC class I cross-presentation pathway for cell-associated antigens by human dendritic cells," *Blood*, vol. 102, pp. 4448–4455, 2003.
[35] C. M. Fehres, W. W. J. Unger, J. J. Garcia-Vallejo, and Y. van Kooyk, "Understanding the biology of antigen cross-presentation for the design of vaccines against cancer.," *Front. Immunol.*, vol. 5, p. 149, 2014.
[36] S. Nierkens, J. Tel, E. Janssen, and G. J. Adema, "Antigen cross-presentation by dendritic cell subsets: one general or all sergeants?," *Trends Immunol.*, vol. 34, no. 8, pp. 361–370, Aug. 2013.
[37] P. Nesmiyanov, "Antigen Processing and Presentation."
[38] J. S. Blum, P. A. Wearsch, and P. Cresswell, "Pathways of antigen processing.," *Annu. Rev. Immunol.*, vol. 31, pp. 443–73, 2013.
[39] P. Nesmiyanov, "Antigen Processing and Presentation | British Society for Immunology." [Online]. Available: https://www.immunology.org/public-information/bitesized-immunology/systems-and-processes/antigen-processing-and-presentation. [Accessed: 04-May-2018].
[40] Health United States, "US National Center for Health Statistics, Leading Causes of Death," 2015. [Online]. Available: https://www.cdc.gov/nchs/fastats/leading-causes-of-death.htm. [Accessed: 04-May-2018].
[41] "World Health Organization, Cancer Key facts, Fact sheets Cancer," 2018. [Online]. Available: http://www.who.int/news-room/fact-sheets/detail/cancer. [Accessed: 04-May-2018].
[42] C. Blanpain, "Tracing the cellular origin of cancer," *Nat. Cell Biol.*, vol. 15, no. 2, pp. 126–134, Feb. 2013.
[43] G. M. Cooper, "The Development and Causes of Cancer," in *The Cell: A Molecular Approach*, 2nd ed., Sunderland (MA):Sinauer Associates, 2000.
[44] D. Hanahan and R. A. Weinberg, "Hallmarks of Cancer: The Next Generation," *Cell*, vol. 144, no. 5, pp. 646–674, Mar. 2011.
[45] Y. A. Fouad and C. Aanei, "Revisiting the hallmarks of cancer.," *Am. J. Cancer Res.*, vol. 7, no. 5, pp. 1016–1036, 2017.
[46] "A Guide to Cancer Surgery | American Cancer Society." [Online]. Available: https://www.cancer.org/treatment/treatments-and-side-effects/treatment-types/surgery.html. [Accessed: 01-Sep-2018].
[47] "Comprehensive Cancer Information - National Cancer Institute." [Online]. Available: https://www.cancer.gov/. [Accessed: 01-Sep-2018].
[48] "Types of Cancer Treatment - National Cancer Institute." [Online]. Available: https://www.cancer.gov/about-cancer/treatment/types. [Accessed: 28-Aug-2018].
[49] "Cancer Immunotherapy." [Online]. Available: https://www.cancer.org/treatment/treatments-and-side-effects/treatment-types/immunotherapy.html. [Accessed: 28-Aug-2018].
[50] L. J. Eggermont, L. E. Paulis, J. Tel, and C. G. Figdor, "Towards efficient cancer immunotherapy: advances in developing artificial antigen-presenting cells," *Trends Biotechnol.*, vol. 32, no. 9, pp. 456–465, Sep. 2014.
[51] J. V Kim, J.-B. Latouche, I. Rivière, and M. Sadelain, "The ABCs of artificial antigen presentation," *Nat. Biotechnol.*, vol. 22, no. 4, pp. 403–410, Apr. 2004.
[52] E. R. Steenblock and T. M. Fahmy, "A comprehensive platform for ex vivo T-cell expansion based on biodegradable polymeric artificial antigen-presenting cells.," *Mol. Ther.*, vol. 16, no. 4, pp. 765–72, Apr. 2008.
[53] C. Wang, W. Sun, Y. Ye, H. N. Bomba, and Z. Gu, "Bioengineering of Artificial Antigen

Presenting Cells and Lymphoid Organs," *Theranostics*, vol. 7, no. 14, pp. 3504–3516, 2017.
[54] H. AN, S. A, S. A1, O. RJ1, 2, and 3, "Artificial Antigen Presenting Cells: An Off the Shelf Approach for Generation of Desirable T-Cell Populations for Broad Application of Adoptive Immunotherapy," *Adv. Genet. Eng.*, vol. 04, no. 03, pp. 1–10, Oct. 2015.
[55] C. J. Turtle and S. R. Riddell, "Artificial antigen-presenting cells for use in adoptive immunotherapy.," *Cancer J.*, vol. 16, no. 4, pp. 374–81, 2010.
[56] L. R. Neal *et al.*, "The Basics of Artificial Antigen Presenting Cells in T Cell-Based Cancer Immunotherapies.," *J. Immunol. Res. Ther.*, vol. 2, no. 1, pp. 68–79, 2017.
[57] B. Prakken *et al.*, "Artificial antigen-presenting cells as a tool to exploit the immune `synapse'," *Nat. Med.*, vol. 6, no. 12, pp. 1406–1410, Dec. 2000.
[58] Q. Ding *et al.*, "RAFTsomes Containing Epitope-MHC-II Complexes Mediated CD4+ T Cell Activation and Antigen-Specific Immune Responses," *Pharm. Res.*, vol. 30, no. 1, pp. 60–69, Jan. 2013.
[59] F. Giannoni *et al.*, "Clustering of T cell ligands on artificial APC membranes influences T cell activation and protein kinase C theta translocation to the T cell plasma membrane.," *J. Immunol.*, vol. 174, no. 6, pp. 3204–11, Mar. 2005.
[60] T. A. Kolesnikova, G. Kiragosyan, T. H. N. Le, S. Springer, and M. Winterhalter, "Protein A Functionalized Polyelectrolyte Microcapsules as a Universal Platform for Enhanced Targeting of Cell Surface Receptors," *ACS Appl. Mater. Interfaces*, vol. 9, no. 13, pp. 11506–11517, Apr. 2017.
[61] "Cytomegalovirus (CMV) Infection - Infections - MSD Manual Consumer Version." [Online]. Available: https://www.msdmanuals.com/home/infections/herpesvirus-infections/cytomegalovirus-cmv-infection. [Accessed: 23-Sep-2018].
[62] J. Picot, C. L. Guerin, C. Le Van Kim, and C. M. Boulanger, "Flow cytometry: Retrospective, fundamentals and recent instrumentation," *Cytotechnology*, vol. 64, no. 2, pp. 109–130, 2012.
[63] M. Brown and C. Wittwer, "Flow cytometry: Principles and clinical applications in hematology," *Clin. Chem.*, vol. 46, no. 8 II, pp. 1221–1229, 2000.
[64] A. Adan, G. Alizada, Y. Kiraz, Y. Baran, and A. Nalbant, "Flow cytometry: basic principles and applications," *Crit. Rev. Biotechnol.*, vol. 37, no. 2, pp. 163–176, 2017.
[65] L. T. Corporation, "Attune ® Acoustic Focusing Cytometer," no. 4453328, 2011.
[66] E. A. Pestana, S. Belak, A. Diallo, J. R. Crowther, and G. J. Viljoen, "Real-Time PCR – The Basic Principles," in *Early, rapid and sensitive veterinary molecular diagnostics - real time PCR applications*, Dordrecht: Springer Netherlands, 2009, pp. 27–46.
[67] M. Arya, I. S. Shergill, M. Williamson, L. Gommersall, N. Arya, and H. R. Patel, "Basic principles of real-time quantitative PCR," *Expert Rev. Mol. Diagn.*, vol. 5, no. 2, pp. 209–219, Mar. 2005.
[68] *Real-time PCR handbook*. .
[69] K. J. Livak and T. D. Schmittgen, "Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method," *Methods*, vol. 25, no. 4, pp. 402–408, Dec. 2001.
[70] T. D. Schmittgen and K. J. Livak, "Analyzing real-time PCR data by the comparative CT method," *Nat. Protoc.*, vol. 3, no. 6, pp. 1101–1108, Jun. 2008.
[71] Sigma-Aldrich, "GenElute ™ Mammalian Total RNA Miniprep Kit User Guide." .
[72] S. D. Gan and K. R. Patel, "Enzyme Immunoassay and Enzyme-Linked Immunosorbent Assay," *J. Invest. Dermatol.*, vol. 133, no. 9, pp. 1–3, Sep. 2013.
[73] "Enzyme Immunoassay and Enzyme-Linked Immunosorbent Assay," *J. Invest. Dermatol.*, vol. 133, no. 9, pp. 1–3, Sep. 2013.
[74] S. PAULIE, P. PERLMANN, and H. PERLMANN, "Enzyme-Linked Immunosorbent Assay," in *Cell Biology*, Elsevier, 2006, pp. 533–538.
[75] J. E. Butler, "Enzyme-Linked Immunosorbent Assay," *J. Immunoassay*, vol. 21, no. 2–3, pp. 165–209, May 2000.

6 Appendixes

Appendix I: Composition of the solutions and reagents used in the work developed

Beads buffer: Solution containing 0.5% of BSA (w/v) and 2mM of EDTA, in PBS 1x

Blocking buffer: solution containing 2% of BSA in washing buffer

PBS 1x: Solution containing 1.47 mM of KH_2PO_4, 4.29 mM of $Na_2HPO_4.7H_2O$, 137 mM of NaCl and mM of KCl, in distilled water (pH=7.4).

Red blood cell lysis 10x: Solution containing 8.02 g of NH_4Cl, 0.84 g of $NaHCO_3$, 0.37 g of EDTA, in distilled water until 100 mL (pH=7.3).

Washing buffer: Solution containing 0.05% (v/v) of Tween 20 in PBS 1x